

SURFACE ENHANCED RAMAN SCATTERING BASED ENDOSOMAL TRACKING
OF GOLD NANOPARTICLES



by
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ABSTRACT

SURFACE ENHANCED RAMAN SCATTERING BASED ENDOSOMAL TRACKING OF GOLD NANOPARTICLES

Understanding the interaction of nanomaterials with living systems is critical to use these novel materials in various fields including nanomedicine. Due to being the first step at the interaction interface, endocytosis has a major role governing the intracellular destinations and behaviors of molecular and non-molecular species including nanoparticles. In this thesis, origin of the intracellular surface-enhanced Raman spectroscopy (SERS) spectra was investigated based on the protein corona structure on the AuNPs surface, endocytosis pathway development and pathway choice of AuNPs. For this purpose, firstly protein corona formation on AuNPs were examined with different cell culture conditions by SERS and effect of the protein corona on the obtained intracellular SERS spectra was investigated. Then, endocytosis pathway choice of AuNPs was investigated with the help of specific inhibitors for each endocytosis pathway. It is aimed to understand whether SERS spectra can tell us endocytosis route into a living cell. For this, endocytosis pathways are inhibited and the obtained SERS spectra are evaluated. Further, the source of the obtained SERS spectra is examined by isolating endosomes and comparing obtained SERS spectra with cellular spectra as time dependent. Finally, the effect of the endocytosis on the macrophage activation and AuNPs phagocytosis is investigated. From the results, it is concluded that protein corona alterations based on different cell culture conditions can be tracked by SERS, and it can affect the intracellular SERS spectra directly. It is also found that SERS can significantly contribute to the investigation of different endosomal pathways, and macrophage activation from single living cells. Furthermore, it is also seen that source of the SERS cannot be thought as only from AuNPs in the endosomes but also from escaped AuNPs, which are evidenced to be found in both cytosol and nucleus accumulated in a time dependent manner. Thus, the origin of the intracellular SERS spectra cannot be considered only from endosomes. Consequently, it is revealed that SERS can distinguish significantly not only protein corona alterations and interference with the intracellular spectra, but also pathways used for nanoparticle uptake and different metabolisms including macrophage activation and phagocytosis.

ÖZET

ALTIN NANOPARÇACIKLARIN ENDOSOMAL YOLAĞININ YÜZEYDE ZENGİNLEŞTİRİLMİŞ RAMAN SAÇILMASI İLE TAKİP EDİLMESİ

Nanomalzemelerin canlı sistemler ile etkileşiminin anlaşılması nanomalzemelerin ilaç ve tıp alanlarında kullanımı için hayati önem taşımaktadır. Bu etkileşimin ilk basamağı olan endositoz ise nanopartiküller gibi birçok molekülün hücre içi yolculuğunun ve böylece hedeflerinin belirlenmesinde oldukça büyük bir öneme sahiptir. Bu çalışmada, hücre içerisinden elde edilen yüzeyde zenginleştirilmiş Raman saçılması (YZRS)'nin kaynağının araştırılması hedeflenmiştir. Bu amaçla altın nanoparçacıkların (AuNP'ler) yüzeyinde oluşan protein korona yapısı, endosomal yolağın gelişimi ve AuNP'lerin hücre içerisine alım yolak çeşitleri incelenmiştir. İlk olarak AuNP'lerin yüzeyinde oluşan protein korona yapıları farklı hücre kültür ortamlarında YZRS ile incelenmiş ve oluşan bu koronanın hücreden elde edilen spektrumlara etkisi araştırılmıştır. Daha sonra ise AuNP'lerin endosomal yolak seçimleri farklı inhibitörlerin kullanımı ile araştırılmıştır. Bu araştırmanın amacı YZRS'nin farklı yolaklar hakkında vereceği bilginin incelenmesi olarak hedeflenmiştir. Ayrıca elde edilen hücre içi YZRS'nin kaynağı zamana bağlı olarak endozomların izole edilmesi ve elde edilen spektrumların tüm hücreden elde edilenler ile karşılaştırılması ile araştırılmıştır. Son olarak ise endositozun makrofaj aktivasyonu ve AuNP'lerin fagositozu SERS ile incelenmiştir. Elde edilen sonuçlardan, protein korona yapısının hücre kültür koşulları ile değiştiği ve bu değişimin YZRS ile izlenebildiği bulunmuştur. Ek olarak elde edilen hücre içi spektruma doğrudan etkisi olduğu saptanmıştır. Ayrıca YZRS'nin farklı endozomal yolakların belirlenmesinde ve makrofaj aktivasyonunun takip edilmesinde kullanılabileceği gözlemlenmiştir. Ek olarak hücre içi YZRS kaynağının sadece endozomların içerisinde bulunan AuNP'lerin değil ayrıca endozomlardan kaçarak sitozol ve ya çekirdekte bulunan AuNP'ler de olabileceği zamana bağlı olarak gözlemlenmiştir. Sonuç olarak, YZRS'nin sadece protein korona yapısındaki değişimleri ve bu değişimlerin hücre içi spektruma etkisini değil aynı zamanda hücre içerisinde nanoparçacıkların alımında kullanılan ya da makrofaj aktivasyonu ya da fagositozunda kullanılan moleküler yolakları da ayırt edebildiği gözlemlenmiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

A549	Human lung carbinoma cell line
Aml	Amiloride
ANOVA	Analysis of variance
AP2	Activating protein 2
ARF	ADP-ribosylation factor
ARH	Autosomal recessive hypercholesterolemia protein
Arp	Actin-related proteins
ATCC	American Type Culture Collection
ATP	Adenine triphosphate
AuNP	Gold nanoparticle
BAR	Bin/amphiphysin/Rvs
Beas-2b	Human bronchial epithelial cell line
BDPs	BAR domain proteins
BMP	Bis(monoacylglycero)phosphate
BSA	Bovine serum albumin
Ca ²⁺	Calcium ion
CaF ₂	Calcium fluoride
CARS	Coherent anti-Stokes Raman scattering
CaME	Caveolae mediated endocytosis
C-C	Carbon carbon bond
CCD	Charge-coupled device
CDC42	Cell division cycle 42
ClME	Clathrin mediated endocytosis
C-N	Carbon nitrogen bond
COPI	Coat protein I
CORVET	Class C core vacuole/endosome tethering
Cpm	Chlorpromazine
C-S	Carbon sulphur bond
CytoD	Cytochalasin D
dG	Deoxy-D-glucose

DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
DMEM-F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
Dyn	Dynasore
D10	DMEM with 10 percent FBS
DF10	DMEM-F12 with 10 percent FBS
D5	DMEM with 5 percent FBS
DF5	DMEM-F12 with 5 percent FBS
EDTA	Ethylenediaminetetraacetic acid
EE	Early endosome
EEA1	Early endosome antigen 1
ECVs	Endosomal carrier vesicles
EHD	Eps15 homology-domain
EM	Electromagnetic enhancement mechanism
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complexes required for transport
FBS	Fetal bovine serum
Fil	Filipin
GAP	GTPase-activating protein
GDF	GDI displacement factor
GDI	GDP dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine-nucleotide exchange factor
GTP	Guanosine triphosphate
HAuCl ₄ .3H ₂ O	Hydrogen Gold (III) chloride trihydrate
HCl	Hydrochloric acid
Hsc	heat shock protein family
HeLa	Cervical cancer epithelium cell line
HEPG2	Hepatocellular carcinoma cell line
iNOS	Inducible nitric oxide synthase
IFN- γ	Interferon gamma

IL	Interleukin
ILV	Intraluminal vesicle
KIF	Kinesin super family protein
LAMP1	Lysosomal associated membrane protein 1
LDA	Linear discriminant analysis
LDL	Low density lipoprotein
LE	Late endosome
LMP	Lysosomal membrane protein
LPS	Lipopolysaccharide
M1	Classically activated phenotype
M2	Alternatively activated phenotype
MHC	Major histocompatibility complex
MVBs	Multivesicular bodies
NA	Numerical aperture
$\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$	Sodium citrate
NaN_3	Sodium azide
NaOH	Sodium hydroxide
NM	Nanomaterial
Nys	Nystatin
Paccin	Protein kinase C and casein kinase substrate in neurons protein 2
PBS	Phosphate-buffered saline
PCA	Principle component analysis
PDMS	Polydimethylsiloxane
Phe	Phenylalanine
PI	Phosphatidylinositol
PIP2	Phosphatidylinositol 4,5-bisphosphate
PI3K	Phosphoinositide 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PI(3,5)P2	Phosphatidylinositol 3,5-bisphosphate
PI(3,4)P2	phosphatidylinositol 3,4-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PNT1A	Normal human prostate cell
PS	Penicillin/Streptomycin

Rab	Ras-associated binding proteins
RAW 264.7	Murine macrophage cell line
RhoA	Ras homolog family member A
SERS	Surface-enhanced Raman scattering
Scr	Sucrose
SNX9	Sorting nexin 9
S-S	Sulphur sulphur bond
SSC	Side scattered light
TGN	Trans-Golgi network
TGF- β	Tumor growth factor beta
TNF- α	Tumor necrosis factor alpha
Trp	Tryptophan
Tyr	Tyrosine
UV-Vis	Ultraviolet/Visible spectroscopy
VAMP2	Vesicle-associated membrane protein 2
WST-8	Water-soluble tetrazolium-8

1. INTRODUCTION

1.1. NANOTECHNOLOGY AND NANOMATERIALS

In 1959, Richard Feynman gave a talk called “There’s Plenty of Room at the Bottom” and he suggested that devices and materials can be fabricated to atomic specifications [1,2]. After that speech, nanotechnology has gained many different definitions and explanations from different institutions and organizations. Dictionary definition of nanotechnology is “controlling size of the materials, devices and systems at the nanoscale for the design, production and application” [3]. The International Standards Organization also gives two definitions as “investigation of the material and processes at the nanoscale which is under 100 nm in one or more dimensions” and “development of improved materials, devices and systems at the nanoscale that differ from the properties of bulk matter and individual atoms, molecules” [4]. US National Nanotechnology Initiative gives a definition as “the ability to work at the molecular level, atom-by-atom, to create large structures with fundamentally new molecular organization and improvement of the novel materials and systems with changed physical, chemical, and biological properties, phenomena, and processes due to their nanoscale size”. As a consequent, European Commission give a nanomaterial definition as a "natural, an incidental, or a produced material including particles agglomerate, aggregate or in an unbound state in which more than 50 percent of them have size range from 1 to 100 nm in one or more external dimensions" [5].

Nanotechnology definition can be covered as a science which includes engineering and application of materials, devices and systems belonging to least at one dimension in the range of 1-100 nm. Reduction to the nanoscale cause changes in electrical, magnetic, morphological, structural, thermal, optical, and mechanical properties of the materials when compared with bulk material [6-9]. These different properties can be controlled by the effects of quantum mechanics rather than classical physics [10].

With the use of nanotechnology concept and nanomaterials in biotechnology, nanobiotechnology concept is raised. Nanobiotechnology become an important field since it has been started to use in almost all areas of biotechnology such as drug delivery,

cell/gene therapy, biomarker development, genomics and proteomics studies, molecular diagnostic, sensor and array development [11-14]. Interactions of nanomaterials with biological molecules, understanding the route of nanoparticles in the biological systems become crucial to understand the fate of the nanoparticles for the use of biotechnological applications. In the case of cellular internalization, endocytosis pathway of nanoparticles is the most important issue that gives opportunity to understand the fate of nanomaterials with toxicological profile and effect on the biological activity of the used nanomaterials. [15].

The interaction of the nanomaterials with biological systems depends not only on the properties of the nanomaterial such as size, shape, surface charge, and hydrophobicity, but also the properties of the biological factors such as surface hydrophobicity, shape, size, conformation of the interacted biomolecule and polarity, pH, viscosity, ionic strength of the interacted microenvironment as seen in Figure 1.1. [5]. For instance, when nanoparticles enter to the human body via inhalation, they interact with lungs and lung surfactant system [16]. When they are administered through intravenous injection, they interact with blood cells, blood proteins and biomolecules [17]. In each type of administration, nanoparticles interact with different biomolecules and microenvironment. Thus, interactions of the nanomaterials will be changed and these interactions should be investigated for each environment.

Internalization through the cell can be both energy dependent or independent [18,19]. Entrance into the cells requires two basic steps as binding (adsorption) to the cell surface which is the slow step and internalization which is the fast step [20,21]. Nanomaterial properties such as size, shape, surface chemistry, surface charge and the cell type affect the interaction including cell surface binding strength [22,23], cellular entrance route [24,25], uptake amount [26,27] and intracellular distribution [28,29].

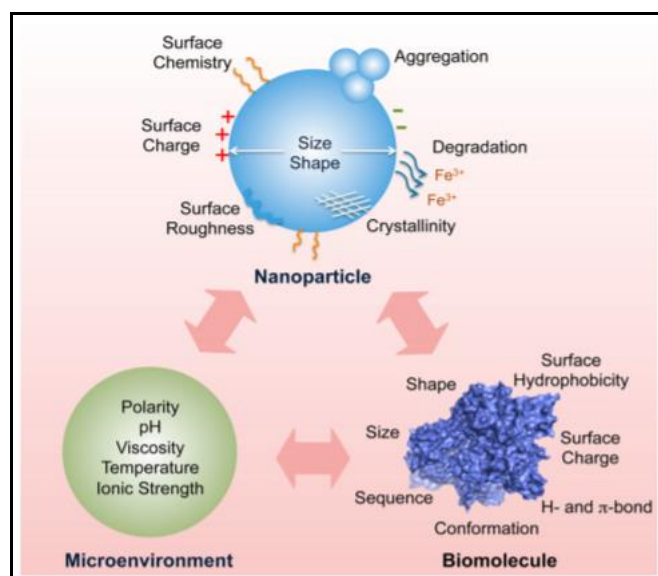


Figure 1.1. Nanomaterial-Biological system interaction with influencing parameters [5]

1.2. ENDOCYTOSIS

Endocytosis is an essential and energy dependent process which is used for communication with the biological environment and internalization of molecules. Internalization of the molecules inside of the cell was first visualized in 1960s using electron microscope [30,31] and then it was realized that it is not only important for uptake of materials but also surface receptor regulation, cell motility, mitosis, control of signaling cascades, pathogen entry, synaptic transmission, receptor down regulation, antigen presentation, cell polarity, growth, differentiation, and drug delivery [15,32-34].

As an expected situation, endocytosis has a relationship with many types of the diseases such as lysosomal storage diseases, Alzheimer's Disease and autosomal recessive hypercholesterolemia [35]. Lysosomal storage diseases refer class of genetic disorders resulting enzyme deficiency. Deficient enzymes are related with the disassembly of many different macromolecules such as lipids, carbohydrates, and proteins in the lysosomes [36]. Absence of that enzymes cause toxic accumulation of undegraded macromolecules in the cell which lead severe or life-threatening conditions [37]. Although there are many different type of treatments including bone marrow transplantation, substrate inhibition, gene therapy and enzyme replacement therapy, all of them has same restriction as rapid removal of the delivered enzyme from the [35,38]. In the Alzheimer's disease, disease

related proteins amyloid precursor protein, β -amyloid protein, and apolipoprotein E are taken by the cell through the endolysosomal trafficking and after internalization, they are sorted to either recycling endosomes or late endosomes. β -amyloid protein is a toxic compound that destroys neurons and it accumulates when amyloid precursor protein is not sufficiently degraded in endosomes [35,39]. As another example, in autosomal recessive hypercholesterolemia, LDL receptor adaptor protein ARH is mutated which is responsible from the interaction between receptor/clathrin and AP2 protein and also recruitment of clathrin coated pits in endocytosis pathway [40]. It is also found that, in myopathies and muscular dystrophies; caveolin3 is mutated [41]. Additionally dysfunction of lysosomes can cause uncontrolled cell growth, improper regulation of cell death and also induction of apoptosis which are the key factors in cancer cell progression [42]. With the understanding of endocytosis process, successful strategies can be developed against many diseases.

In a mammalian cell, 50-180 percent of the surface area of the cell membrane is cycled in and out of the cell every hour and the amount of internalized fluid corresponds to 30 percent of the total cell volume per hour of which about two thirds are returned to the extracellular space within about 10-15 min [43,44]. Endocytic pathway includes 3 pathways in fundamental. First one is a recycling pathway for plasma membrane components and ligands. Second pathway is degradative pathway for the degradation of internalized molecules. Last pathway is a connecting unidirectional feeder pathway for transport of fluid and membrane components from the recycling pathway to the degradative pathway as seen in Figure 1.2. [2].

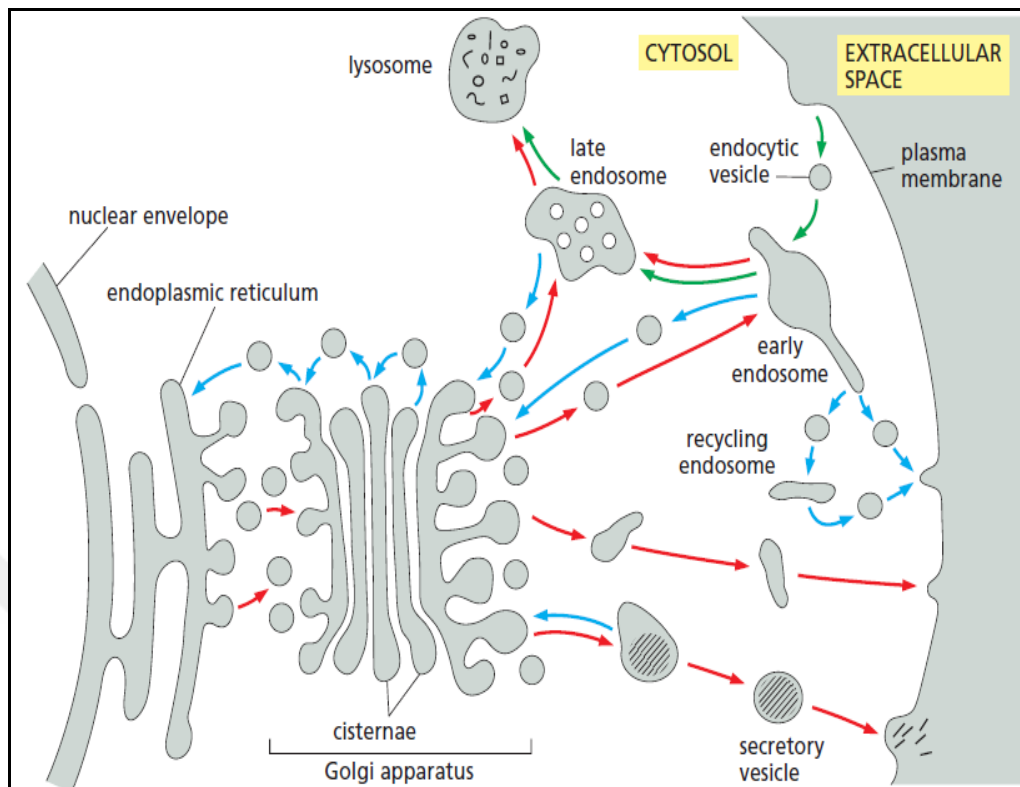


Figure 1.2. Endosomal trafficking. Secretory pathway is shown with red arrows; endocytic pathway is shown with green arrows and retrieval pathways including recycling of endosomes are shown with blue arrows [45].

When mechanochemistry of the endocytosis is taken into consideration; endocytosis proceeds in two steps as invagination of the plasma membrane and pinching off of the vesicle. Generally, cell cortex is resistant to deformation which means shape changes require large amount of energy [46]. For the endocytosis, cell needs overcome resistant to deform the membrane which is regulated by the pulling and pinching forces. Five components generating pulling and pinching forces were determined [47]. The first components are phosphoinositides (PI) which cover the endocytic membrane and used for the recruitment of the endocytic proteins [48-51]. The second components are coat proteins which differ according to the type of the endocytosis but generally used for anchor and regulation of actin filaments for membrane curvature and budding [52]. The third components are proteins which accumulate in the endocytic site such as BAR domain proteins (BDPs) and used for the regulation of membrane deformation and curvature [53]. Forth component is the cytoskeleton which provides not only pushing forces for bringing closer together of neck membranes but also pulling forces to keep the neck under tension.

This tension provides ability of dynamin fission for dynamin dependent endocytosis [54, 55]. Fifth components are the enzymes that hydrolyze PIs. Hydrolysis rate of PIs alter local membrane curvature and maturation of endosomes [56].

When first step of the endocytosis starts at $t \sim 0-20$ s, membrane deformation is started by the accumulation of coat proteins with a generation of a small dome sized less 50 nm. Then, the nucleation factors are recruited to the site and activated for actin polymerization. This obtained dome shape is the prerequisite for further deep invagination of the membrane because it creates a suitable angle for F-actin pulling force upon the bud region. At this time, phosphatidylinositol 4,5-bisphosphate (PIP_2) accumulates uniformly all over the endocytic site with the help of the enzymes which phosphorylate lipid precursors [47].

At $t \sim 20-25$ s, coat proteins recruited for the F-actin polymerization. With the initiation of F-actin polymerization, pulling force increases to drive further membrane invagination [47]. When membrane invagination starts, actin monomers integrate with existing actin filaments from their barbed ends [57]. At that site, myosin pushes the actin network through the cytoplasm as contractile force and with the pushing force, obtained invagination provides favorable binding site for dynamin. With the dynamin binding, it deforms the adjacent membrane as an optimal curvature for the accumulation of more dynamin. Meanwhile, PIP_2 levels are decreased by phosphatases and dynamin protect PIP_2 from hydrolysis at the bulb. Although PIP_2 hydrolysis is protected at the bulb, they are hydrolyzed on the bud and this led to the lipid phase segregation at the dynamin-coat protein boundary. Differences in the PIP_2 levels at neck/bulb juncture generate a circumferential force which acts like a noose [58]. This separation and PIP_2 level differentiation lead generation of the interfacial force which is resulted as the neck formation [47].

At $t \sim 21-27$ s, obtained interfacial force grows sharply and this results not only squeezes of the neck but also more PIP_2 hydrolysis and this cause increase in the interfacial force and more squeeze of the neck as a self-accelerating process.

At $t \sim 29$ s, obtained interfacial force narrows the neck down to <5 nm which provides fusion of the opposite bilayers spontaneously [59] and then rapid vesicle scission. When vesicle is bud from the membrane, dynamin disassemble from the bulb within 3s. When dynamin started to disassemble, PIP_2 starts to hydrolyze in both bulb and bud region. With

the PIP₂ phosphatase activity, coat proteins start to disassemble, F-actin attachment to the bud starts to weaken and at the end, pulling force is lost. In that point, pinching force is caused by the differential phosphatase activity along the membrane invagination [47].

After the detachment, vesicle delivers its cargo to other subcellular compartments after 1-5 min from the internalization of cargo [15,60] which is responsible of three main processes as (i) ensuring the recycle of housekeeping receptors back to the plasma membrane (directly or indirectly via recycling endosome), (ii) sorting material toward the trans-Golgi network (TGN) and (iii) shuttling receptors and internalized materials that require down regulation/degradation to the late endosome (LEs). When early endosome (EE) internalized cargo and fluid, fluid is recycled away rapidly and cargo remained and accumulated. [61]. EE is rich in Rab5 GTPase with its effectors VPS34/p150, phosphoinositide 3-kinase (PI3K) and its product phosphatidylinositol-3-phosphate (PI3P) [15,62-64]. Rab5 is the most important marker for EEs found from the beginning of the formation of EEs and it determines the functions of EEs and the maturation to the LEs by its guanine-nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), GDP dissociation inhibitors (GDIs), GDI displacement factors (GDFs) [65,66]. EEs have a continuous communication with the TGN which is required for the maturation process, arrival of hydrolases and removal of endosomal components during maturation [2]. EEs are heterogenous compartments based on their morphology, localization, composition and also function and their distribution is cell-type dependent [67]. EEs have complex structure with tubular and vacuolar domains. Most of the membrane surface area is in the tubules and much of the volume in the vacuoles. The limiting membrane consists of a mosaic of subdomains with different function and composition and they include domains enriched in Rab5, Rab4, Rab11, Arf/COPI, retromer [68].

The formation of intraluminal vesicles (ILVs) is started in EEs with the EEs characteristic 'plaques' containing clathrin and components of the endosomal sorting complex required for transport (ESCRT) [69]. EEs often consists of several ILVs, for instance there are 1-8 ILVs in the EEs of the HepG2 cells [70]. EEs have pH between 6.8-5.9 and have relatively low Ca²⁺ concentration [71]. ILVs are the most important component not only for the formation and maturation of LEs but also for the selective sorting of the cargo for the degradation. ILVs are formed because signaling receptors are inactivated in ILVs due to the contact loss with cytosol. On the other hand, lipids and proteins are delivered to the

lysosomes more easily with ILVs for hydrolysis. The lipid structures of the ILVs differ from the limiting membrane. ILVs contain enriched phospholipid bis(monoacylglycerol) phosphate/lysobisphosphatidic acid (BMP/LBPA), which is negatively charged and contributes to the recruitment of positively charged lipid hydrolases, cholesterol, sphingolipids, PI3P, and ceramide [72,73]. With the maturation, concentration of BMP/LBPA and ceramide increase and concentration of cholesterol decreases due to extraction and transport to the ER with the action of NPC1 and NPC2 [74]. PI3P is also lost in the later phases due to the degradation and modification by kinases/phosphatases [75].

EEs have slow, short-range movements and when they mature, movements become long-range oscillations with a net movement towards the perinuclear region where lysosomes are localized. These movements are regulated by dynein and kinesin motors which provide opposite direction movement of endosomes [76,77]. Dynein and kinesin activity is required for the fusion of endosomes each other and fission of EEs and cargo sorting into degradative and recycling pathways [78,79]. Kinesins such as KIF16B and KIF3A are large family of plus-end-directed motor proteins. KIF16B attaches EEs through PI3P when Rab5 is activated and used for the plus-end directed EEs movement. Furthermore, it changes the balance between degradation and recycling of cargo [80]. Depletion of KIF16B causes a block of cargo recycling and overexpression cause increase in the cargo degradation. On the other hand, KIF3A is used for LE trafficking [81]. LEs movement for the fusion with lysosomes are generally dynein-dependent [82]. LEs bind to the dynein with Rab7 activity [83]. With Rab7 activation, Rab7 binds its effectors and recruit dynactin subunit and this interaction provide minus-end-directed movement of LEs. Cholesterol has also role in the movement of LEs which is associated with the LE binding with dynactin complex [84]. On the other hand, actin cytoskeleton is also used for the movement of endosomes. It is known that Arp2/3 driven actin nucleation is required for LE-lysosome fusion, sorting and vesicle fission in the recycling pathway, cargo transport to the TGN and lysosomes.

LEs have 250-1000 nm size generally with low density and more acidic nature than EEs and it receives endo-lysosomal proteins in between endosomal pathway route from TGN to lysosomes. Levels of recycling proteins and high levels of degradation regulatory proteins are characteristic for LE and it consists of tubular and multivesicular areas in ILVs [60].

The limiting membrane contains cholesterol and sphingolipid-rich lipid rafts and membrane protein aggregates such as LAMP1 and lumen contains acid hydrolases and more than 30 ILVs with 50-100 nm sizes.

The formation of LEs is started with the Rab7 domain formation for the switch from Rab5 to Rab7. This formation causes the transient Rab5/Rab7 endosome hybrid. After Rab7 is recruited by Rab5, Rab5 is converted to the GDP-bound form and dissociates with its effectors within a few minutes and it is replaced by Rab7 [85]. Another mechanism explains switch process by a fission which separates Rab7 domain from the rest of the hybrid endosome structure containing Rab5, Rab4, and Arf1 [86,87]. Switch of Rab5 to Rab7 is a fundamental step for the LEs formation and maturation. Removal of Rab5 requires inhibition of the feedback loop for more Rab5 binding and also GTPase-activating protein (GAP) activity to provide GTP hydrolysis activity of Rab5. For the termination of Rab5 function on endosomes, a GAP has to activate Rab5-mediated hydrolysis of bound GTP into GDP such as RN-Tre and RabGAP5 [63,88]. After switch of the Rab5 to Rab7, fusion process of LEs altered, with that alteration; LEs can fuse only with other LE and lysosomes. Fusion mechanism is regulated by class C VPS CORVET for EEs and HOPS complexes for LEs [89] and conversion of CORVET/HOPS is connected to the Rab5/Rab7 conversion [2].

In endosome maturation; PI conversion is another key point like Rab5/Rab7 switch. PI3P and phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂) are among the most important PIs for endosome maturation and they identify the EE and LE membranes. PI3P is found on the cytosolic leaflet of EE membranes formed by VPS34 which is a phosphoinositide 3-kinase (PI3K) [90]. VPS34 is recruited by Rab5-GTP. VPS34 forms a complex with p150 and Beclin-1 which are used for the regulation of kinase activity of the VPS34 [91]. Formed complex is associated with UVRAG protein which is an activator of the HOPS complex. With the activation of HOPS complex, Rab5/Rab7 switch can be achieved. PI(3,5)P₂ is used in the degradative pathway. It is formed by phosphatidylinositol 3-phosphate 5-kinase (PIKfyve). PIKfyve activity is regulated by ArPIKfyve/Vac14p and the PI(3,5)P₂ phosphatase Sac3/Fig4p which are the activators of it and they together form a stable complex important for lipid kinase and phosphatase activities [92]. PIKfyve is associated also with the synthesis of phosphatidylinositol 5-phosphate (PI5P) and p40 an effector of Rab9 that this activity is important for the transport of LE to the TGN [93].

After the maturation of EEs to the LEs, newly formed LEs continue maturation process with exchange of membrane components, change in the fusion partners, formation of additional ILVs, and drop in the luminal pH, acquisition of lysosomal components and change in morphology and movement to the perinuclear area.

The pH decrease in the LEs up to 4.5 provides optimal environment for hydrolytic reactions and also important for membrane trafficking, sorting and routing of cargo, inactivation of entered pathogens, etc. An asymmetric pH between the TGN and LEs allow receptors to bind ligands in one compartment and release from the other one [94]. pH decreases rapidly about 0.5 pH units after LE formation and continues decreasing until fuse with lysosomes. Luminal pH is regulated by V-ATPase concentration in the membrane, selection of V-ATPase isoforms [62,95]. V-ATPases generate a positive charge inside the membrane potential, it affects Na^+/K^+ ATPase, Cl^- , Ca^{2+} , Na^+ , and K^+ ion influx and efflux which are important for fusion with lysosomes and reformation of lysosomes from endoysosomes [96].

When LEs move to the perinuclear area, they fuse with each other to form larger bodies and undergo transient ('kiss-and-run') fusions [97] and if endocytosis pathway is associated with the lysosomal degradation, internalized cargo reaches the lysosome for the degradation after 30 min from the internalization [15,60] while if it is not associated with the lysosomal degradation, intravesicular contents recycle back to their original membrane domain from sorting endosomes. When LEs are fused with lysosomes, most of its components are degraded in the lysosomal environment.

Lysosomal compartment consists of vacuoles and have heterogeneous structure for composition, morphology, location and density like EEs. They have high density and their heterogeneous structure is not only caused by the different type of cargos but also cargo degradation processes in the individual vacuoles and fusion processes between the vacuoles. Lysosomal function is regulated by soluble lysosomal hydrolases and integral lysosomal membrane proteins. There is nearly 50 identified lysosomal hydrolases and each of them has their own specialized substrates. They are responsible for the lysosomal catabolic capacity [60]. Identified nearly 25 lysosomal membrane proteins are responsible for acidification, protein import and export and membrane fusion. With the use of responsible proteins, internalized cargos are degraded by lysosomes [15]. Development of

the endosomal pathway is shown in Figure 1.3. and properties of the early and late endosomes can be seen in Table 1.1.

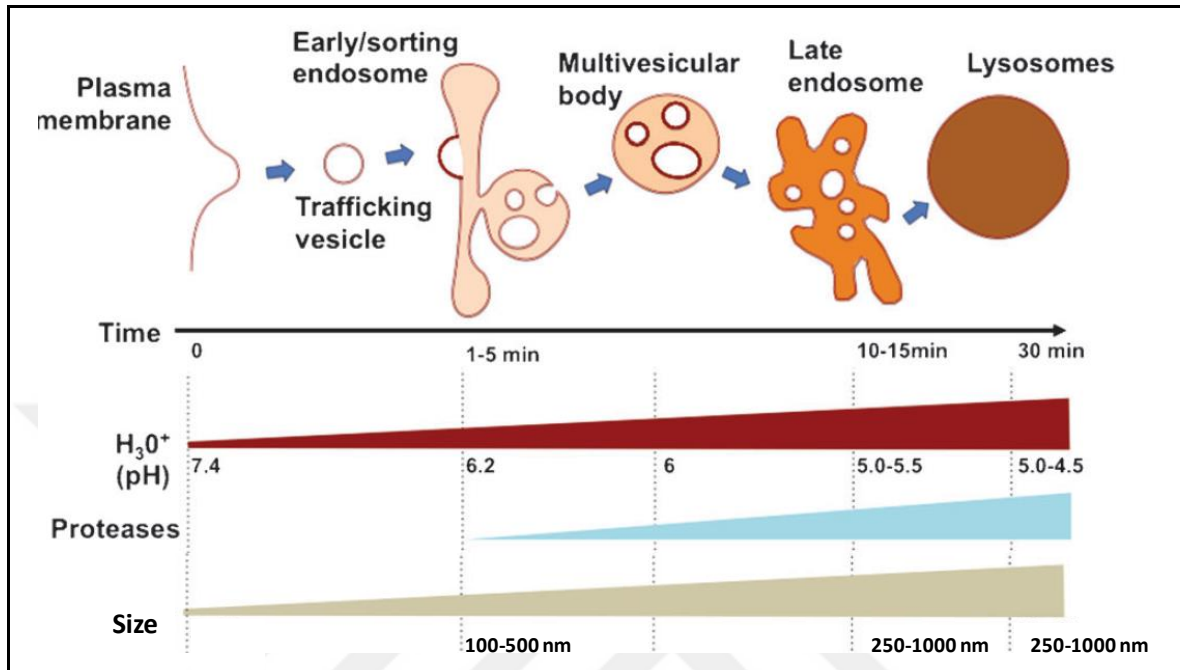


Figure 1.3. Endosomal pathway maturation with its properties [15]

Table 1.1. Properties of Endosomes [98]

Properties	Early Endosomes	Late Endosomes
Topology	Peripheral to perinuclear	Perinuclear
Morphology	Tubular	Complex, internal membranes
Luminal pH	6.2-6.5	5.5
GTP-binding proteins	Rab4, Rab5	Rab7, Rab9
Peripheral Proteins	EEA1, annexin II	Annexin I

1.2.1. Endocytosis Pathways

Although many mechanisms related with endocytosis are known; it is still unclear why different endocytic pathways are required for different cargoes. This can be caused by the differential requirements for many parameters such as speed, cell signaling, delivery type of cargo, and membrane area/lipid turnover [32]. On the other hand, all uptake mechanisms have same stages. First, cargo interacts with cell surface and provides specific binding. Second, cargo is engulfed in membrane invagination which continues with plasma membrane budding, pinch off from membrane and formation of vesicles known as endosomes. Third, endosomes deliver the cargo to the specialized vesicular structures which enables sorting of cargo towards different destinations by membrane trafficking. Finally cargo is delivered to the subcellular organelles, and recycled [15,99]. Uptake pathways can be classified in two major groups as phagocytosis (cell eating) and pinocytosis (cell drinking) [15]. Phagocytosis is specialized type of actin-dependent endocytosis used by immune cells for uptake of pathogens, dead cells, and cell debris [15,100]. It also used for the degradation of particles larger than 0.5 μm [34]. Pinocytosis is used to internalize fluid surrounding the cell to uptake of all substances in the fluid phase [34]. It can be used for all type of cells and has multiple forms depending on the cell origin and function. It can be classified as clathrin dependent and clathrin independent endocytosis. Clathrin independent endocytosis includes caveolae dependent endocytosis, clathrin and caveolae independent endocytosis and macropinocytosis. Clathrin and caveolae independent endocytosis has also subgroups as Arf6, flotilin, Cdc42 and RhoA dependent endocytosis (Figure 1.4.) [99]. Clathrin dependent, caveole dependent, and clathrin and caveolae independent endocytosis are also classified as receptor mediated endocytosis and they have the advantage of rapid ligand targeted internalization [100] and ability to bypass potential effects of the multi-drug resistance associated efflux transporters which are upregulated in tumor tissues [35,101].

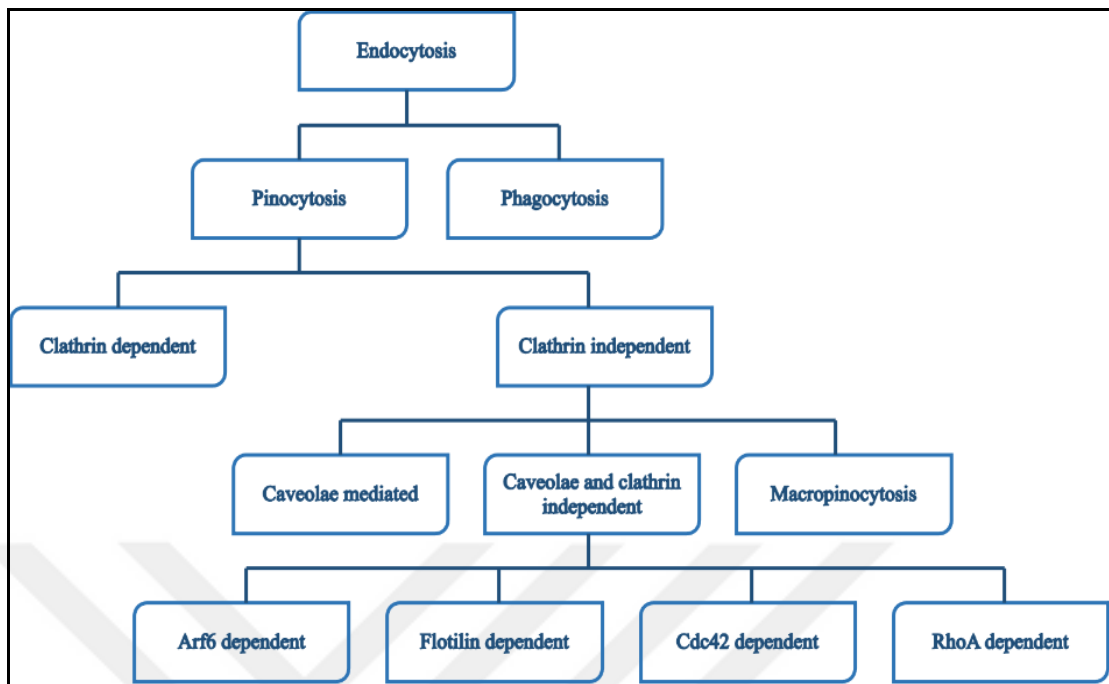


Figure 1.4. Classification of endocytosis pathways [99]

1.2.1.1. *Phagocytosis*

Phagocytosis is actin-dependent mechanism that is used for the internalization of particles larger than 0.5 μm . Lower organisms use phagocytosis as primer internalization pathway for nutrient while it is used by specialized phagocytic cells such as macrophages and neutrophils for defense mechanism. Thus, phagocytosis by macrophages is extremely complex and critical for the uptake and degradation of pathogens, dead cells and it also plays critical roles in tissue development and remodeling, immune response and inflammation. In phagocytosis, large varieties of receptors are used and most of the particles are recognized by more than one receptor and these receptors have ability to cross-talk and synergy for recognition. Moreover, most of them have dual functions such as adhesion and internalization [30].

Beyond the complexity, phagocytosis can be explained with following steps: 1- Internalization is initiated by the interaction of receptors of phagocyte with ligands of particle. 2- This interaction leads to the actin polymerization which provides formation of phagosome. 3- After internalization, actin is depolymerized from the phagosome and the phagosome starts to maturation with the formation of early phagosome, then matures to

the late phagosome and end with the formation of phagolysosomes similar to the endosome maturation (Figure 1.5.) [30].

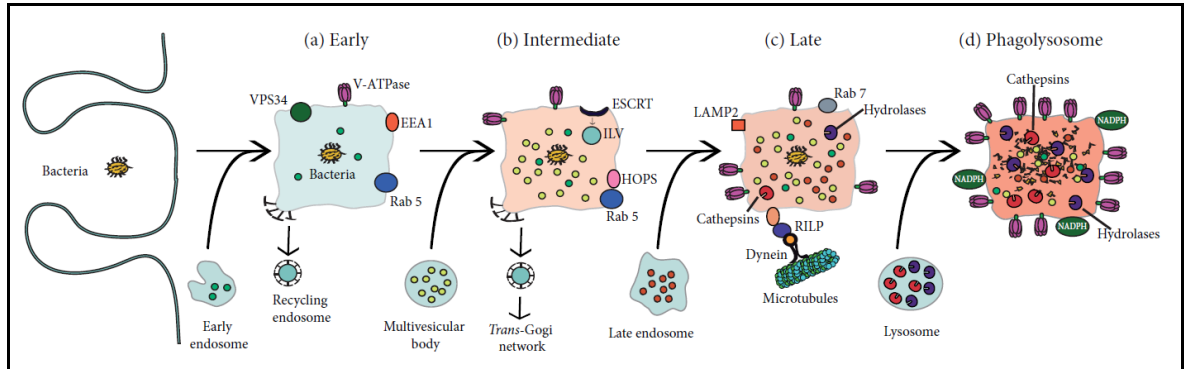


Figure 1.5. Phagocytosis maturation process with the formation of (a) early phagosome, (b) intermediate structure, (c) late phagosome and (d) phagolysosome [102].

As a first step, phagocytosis starts with the recognition of cargo, which can be either foreign particles or apoptotic cell components.

For the recognition of foreign particles, used receptors can be divided into two groups as non-opsonic and opsonic receptors. Non-opsonic receptors, which are also called as pattern-recognition receptors, generally detects pathogen-associated molecular patterns (PAMPs) which are unique molecules found in bacteria, fungi, and parasites while never found in higher organisms [103]. These type of patterns can be detected by many different non-opsonic receptors including Toll-like receptors (TLRs), mannose receptor (CD206) [104], dectin-1 [105], CD14 [106], scavenger receptor A [107], and CD36 [108]. Foreign particles can be also recognized by soluble molecules in the blood while circulating and in interstitial fluids. These soluble molecules, which are called opsonins, bind to the foreign particles and tag them for the recognition by opsonic receptors. Immunoglobulins and complement components are among important opsonins and they can directly induce efficient phagocytosis while most of the non-opsonic receptors could not induce phagocytosis directly. Fc γ receptors (Fc γ R) are among the most important opsonic receptors which are glycoprotein family expressed on leukocytes membrane and they can bind to the Fc portion of the immunoglobulin G (IgG) molecules including subclasses of IgG [109]. Along the Fc γ receptors; complement receptors (CRs) are also important

opsonic receptors which recognize components of the complement cascade which is deposited on the surface of foreign particles [110].

For the detection of apoptotic cells, specific molecules which are released by cells in apoptosis and also molecules displayed on the surface of the apoptotic cells such as phosphatidylserine (PS) are recognized by different receptors. T cell immunoglobulin muvin (TIM) family receptors [111], stabilin-2 [112], and brain-specific angiogenesis inhibitor 1 (BAI-1) [113] receptors can directly recognize PS and some of the receptors recognize a complex of released molecules bonded to PS. For instance, lactadherin (MFG-E8) can connect PS to $\alpha V\beta 3$ integrins [114]. On the other hand scavenger receptors A (SR-A), MARCO and CD36 can also detect apoptotic cells [115].

When a particle is interacted with phagocyte receptors, signaling events are triggered for the activation of the phagocytosis. Fc γ signaling [116], lipid signaling [117], small GTPase [118] signaling, and complement receptor signaling [119] provides trigger for phagocytosis activation. After activation, pseudopods which cover the target particle are formed with the help of membrane remodeling and actin cytoskeleton. Then, phagocytic cup, which is depression of the membrane, is formed and membrane surrounds the target particle and with the closure of the membrane, phagosome is formed.

After phagocytic receptors recognize target ligands and aggregate to initiate signaling pathways, actin cytoskeleton is regulated for the membrane protrusions and formation of pseudopodia. As the first step of actin remodeling, membrane-associated cortical cytoskeleton is disrupted, then F-actin polymerization is initiated with actin filaments nucleation and pseudopodia is extended. As a last, actin depolymerization occurs for the formation of phagosome [120]. After the formation of phagosome; it matures and lastly form phagolysosomes like the endolysosomes for the destruction of target particles. Maturation process starts with the early phagosome formation which has same properties with early endosomes [121]. Early phagosome is obtained by membrane fusion between phagosome and early endosomes. Membrane fusion is regulated by the small GTPase Rab5 [122] which recruits early endosome antigen 1 (EEA1) to promote phagosome-early endosome fusion [123], and Rab5 also recruits class III PI3K human vacuolar protein-sorting 34 (hVPS34), which generates PI3P. With the formation of PI3P; EEA1 can recruit other proteins required for the phagosome maturation such as Rab7 which is late endosome

marker [124]. After the early phagosome formation, lumen is acidified by the accumulation of V-ATPases on the phagosome membrane to form late phagosomes. For the maturation, Rab5/Rab7 conversion is achieved, proteins required for former steps are recycled back, and ILVs are formed. Once Rab7 recruited, it also recruits proteins required for maturation. Rab-interacting lysosomal protein (RILP) recruited for binding with dynein-dynactin complex [125] to contact with microtubules. With the phagosome-microtubule contact; phagosomes moved in close contact to lysosomes which are going to be fused to form phagolysosomes. Fusion is achieved by soluble NSF attachment protein receptor (SNARE), vesicle-associated membrane protein (VAMP) 7, VAMP8 [126], lysosomal-associated membrane proteins (LAMPs) and luminal proteases [127]. Phagolysosomes have PI3P enriched internal membrane [128] and contain hydrolytic enzymes including cathepsins, proteases, lysozymes, and lipases [126]. Besides the hydrolytic enzymes, other microbicidal components also help for the digestion of target particle. For instance, NADPH oxidase generates superoxide (O_2^-) which generate reactive oxygen species (ROS) including hydroxyl radicals and singlet oxygen [129].

1.2.1.2. Pinocytosis

Pinocytosis is used to internalize fluid surrounding the cell to uptake of all substances in the fluid phase and classified as clathrin dependent and clathrin independent endocytosis. Clathrin independent endocytosis includes caveolae dependent endocytosis, clathrin and caveolae independent endocytosis and macropinocytosis. Clathrin and caveolae independent endocytosis has also subgroups as Arf6, flotilin, Cdc42 and RhoA dependent endocytosis.

Clathrin Mediated Endocytosis (CME)

Clathrin mediated endocytosis is among the most important entry mechanisms for the internalization of nutrients which are smaller than 300 nm, and also for the receptor signaling [15,130]. In the first step of clathrin dependent endocytosis, trans membrane receptors (transferrin receptor and low density lipoprotein receptor) are sorted and they bind to their ligands (transferrin and low density lipoprotein), then adaptor protein AP2 recruited and it binds to the endocytic motifs in cytoplasmic tails of receptors [131] and plasma membrane-specific lipid PIP2 [132] (Figure 1.6.).

AP2 is a heterotetramer with two large subunits α and $\beta 2$ and two smaller chains $\mu 2$ and $\sigma 2$. Medium chain ($\mu 2$) of AP2 is responsible of the recognition of tyrosine-based endocytic sorting motifs of multiple cargo proteins [133]. α subunit binds to the PIP2 and PIP3 to regulate AP2 self assembly [134] and interaction with tyrosine based endocytosis motifs [135].

Clathrin coated pit formation starts with the binding of AP2 and cargo-specific adaptor proteins on the cytosolic side of the plasma membrane with the lifetime between 20 s and 2 min. This coated pit is originated from cytosolic proteins, especially from clathrin. Clathrin is three legged structure known as triskelion which provides assembly of the three clathrin heavy chains, each associated with a clathrin light chain [136,137] (Figure 1.6.a). At physiological conditions, clathrin triskelions self assemble into cages in the presence of adaptor/accessory proteins such as adaptin, amphipysin, endophilin, Eps15, intersectin and actin (Figure 1.6.b) [138,139]. Adaptin provides polymerization of clathrin subunits to form polyhedral lattice scaffold with binding to cell surface receptors on the extracellular face as well as binding to clathrin heavy chains on the cytosolic side. Endophilin, Eps15 and intersectin provide connection of endosomal structures with actin cytoskeleton [140,141]. Endophilin interacts with dynamin and also actin for dynamin recruitment and provide linkage with cytoskeleton [142]. Amphiphysin interacts with dynamin, clathrin and also AP2 for membrane deformation, dynamin recruitment, and connection between membrane and cytoskeleton [143-145]. Actin brings surrounding membrane into close proximity which defines spatial regulation and movement of endocytic vesicle.

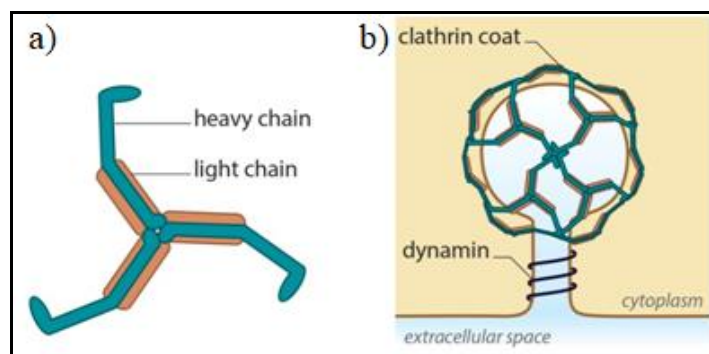


Figure 1.6. Structure of a) clathrin, b) clathrin coated vesicles [146]

Assembled vesicle is pinched off from the plasma membrane with the help of a GTPase dynamin, which is recruited by BAR-domain containing proteins such as amphiphysin, endophilin and sorting nexin 9 (SNX9). Recruited dynamin assembles around the neck of the budding vesicle and causes scission and intracellular release of vesicle [35,147]. GTPase activity is stimulated by dynamin binding to PIP2 or PIP3 [148] and with the hydrolysis of GTP by oligomeric rings of dynamin around the neck; scission of the vesicle is occurred by the help of two forces provided by dynamin: pulling force to bring neck of the membrane into close apposition and also a twisting force on tubular membranes [55]. Scission of the vesicle can occur by direct constriction [149] ('pinchase'), by helical expansion of dynamin spirals at the neck [150] ('poppase') or with the help of downstream effectors [151] such as lysophosphatidic acid acyl transferase endophilin (Figure 1.7.) [152,153].

After coated vesicle is formed as seen in Figure 1.7., accessory proteins disassemble and recycled back to the cytoplasm and lumen starts to acidify up to 6.8 due to the active transport of H^+ by the V-ATPases incorporated into the coated vesicle [154]. Then, clathrin coat is quickly shed after intracellular invagination by an uncoating protein heat shock protein (hsc70) family with its cofactor auxilin which provides movement of the vesicle and fusion with targeted endosome; early endosome [155-157]. In the early endosomes, intra-organellar acidification continues up to pH 6.5. With pH decrease, some receptors dissociated from their ligands and activation of hydrolytic enzymes is started. Rab5 and Rab4 regulate trafficking through the early endosome.

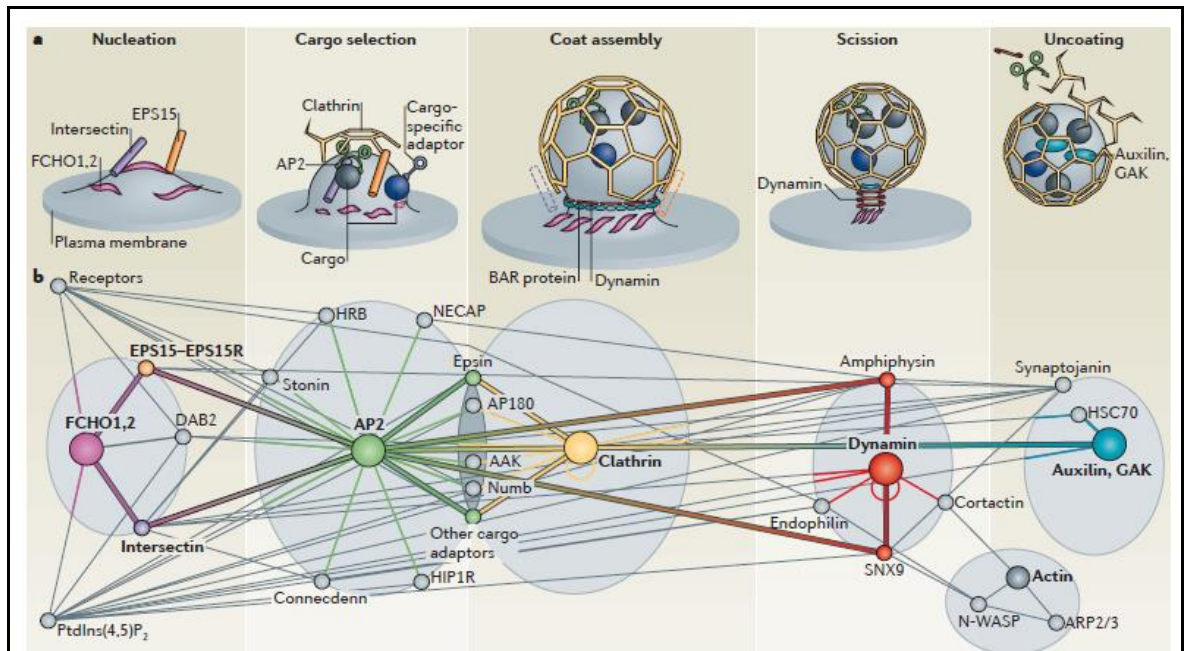


Figure 1.7. CIME a) maturation, b) protein interactions [158]

Then, the endosomes move along the cytoskeleton to fuse with early or sorting endosomes with the help of microtubules, microfilaments, and molecular motor proteins. In this step, the intravesicular contents of the endosome carried with the endolysosomal pathway for degradation in the lysosomes [159]. Endosomal carrier vesicles detach from the early endosome, form multivesicular bodies (MVBs) and then mature into late endosomes. In the maturation process, pH decreases (pH 6.2-5.2 for late endosome/multivesicular body, pH 5.2-4.5 for lysosome) and it creates an environment conducive to enzyme function. pH decrease activates acid hydrolases which are retained in prelysosomal vesicles that will fuse with late endosomes, creating endolysosomes, where macromolecular degradation occurs. Lysosomes have 4.5-5 luminal pH maintained by proton pumping V-ATPases. Main regulators of the function are soluble lysosomal hydrolases which are proteases, lipases, phosphatases, sulfatases, and lysosidases and integral lysosomal membrane proteins (LMPs). They have roles in acidification, protein import/export and membrane fusion [160]. The major role of lysosome in the endocytosis is degradation of the internalized cargo [161-163].

Caveolae Mediated Endocytosis (CaME)

Although clathrin-dependent endocytosis is the most preferred pathway for endocytosis in most cells, caveolae mediated endocytosis is the most studied clathrin independent pathway which provides endocytosis of particles smaller than 80nm and cell signaling, lipid regulation, and vesicular transport (Figure 1.8.) [164].

Caveolae were first identified in 1950 based on their unique morphology observed by electron microscopy [165,166]. Caveolae are 50-80 nm large flask-shaped invaginations displayed by the plasma membrane of cells which are the cholesterol and sphingolipid rich plasma membrane regions (lipid rafts) [167,168]. Large head groups of the sphingolipids provides required space filled by cholesterol [32].

Caveolae have a striated coat on the surface of the membrane formed by specially caveolin-1. Caveolin-1 is the characteristic hairpin-like membrane protein and responsible for the structural formation of the caveolae. [15]. There are also other proteins that are used for the caveolin dependent endocytosis such as cavin, which induces membrane curvature, dynamin, which enables vesicle scission, vesicle-associated membrane protein (VAMP2) and synaptosome-associated protein (SNAP), which mediate subsequent vesicle fusion and actin which provides not only the movement of newly formed vesicle through the cortex of the plasma membrane but also the formation of the caveolar vesicles [169-171]. With the help of the dynamin; caveolae coated vesicles bud off from the plasma membrane, transport and fused with caveosomes or MVBs with the help of actin [172,173]. Membrane of obtained vesicles is rich in cholesterol and glycosphingolipids and they have neutral pH [174]. Regulation of CaME is controlled by different proteins such as EHD2, which is an ATPase, and F-BAR-domain protein pacsin2. EHD2 is a negative controller used for the interaction with actin cytoskeleton [175,176]. EHD2 also interacts with Pacsin2 to bind with dynamin and caveolin-1 and regulate membrane curvature [177].

Unlike to the clathrin dependent endocytosis; endosomes, lysosomes, and TGN do not participate the trafficking in caveolae dependent endocytosis; thus internalized cargos are delivered to non-lysosomal subcellular compartments [178]. Caveosomes are transported along microtubules to perinuclear membrane organelles directly which are identified as smooth ER. During travel of caveosome, pH is maintained as neutral and caveosome do not fuse with lysosomes; instead of this, cargo is delivered directly to ER [179,180].

Although it is slower pathway for endocytosis, it can bypass lysosomal degradation that can be used as an advantage [99].

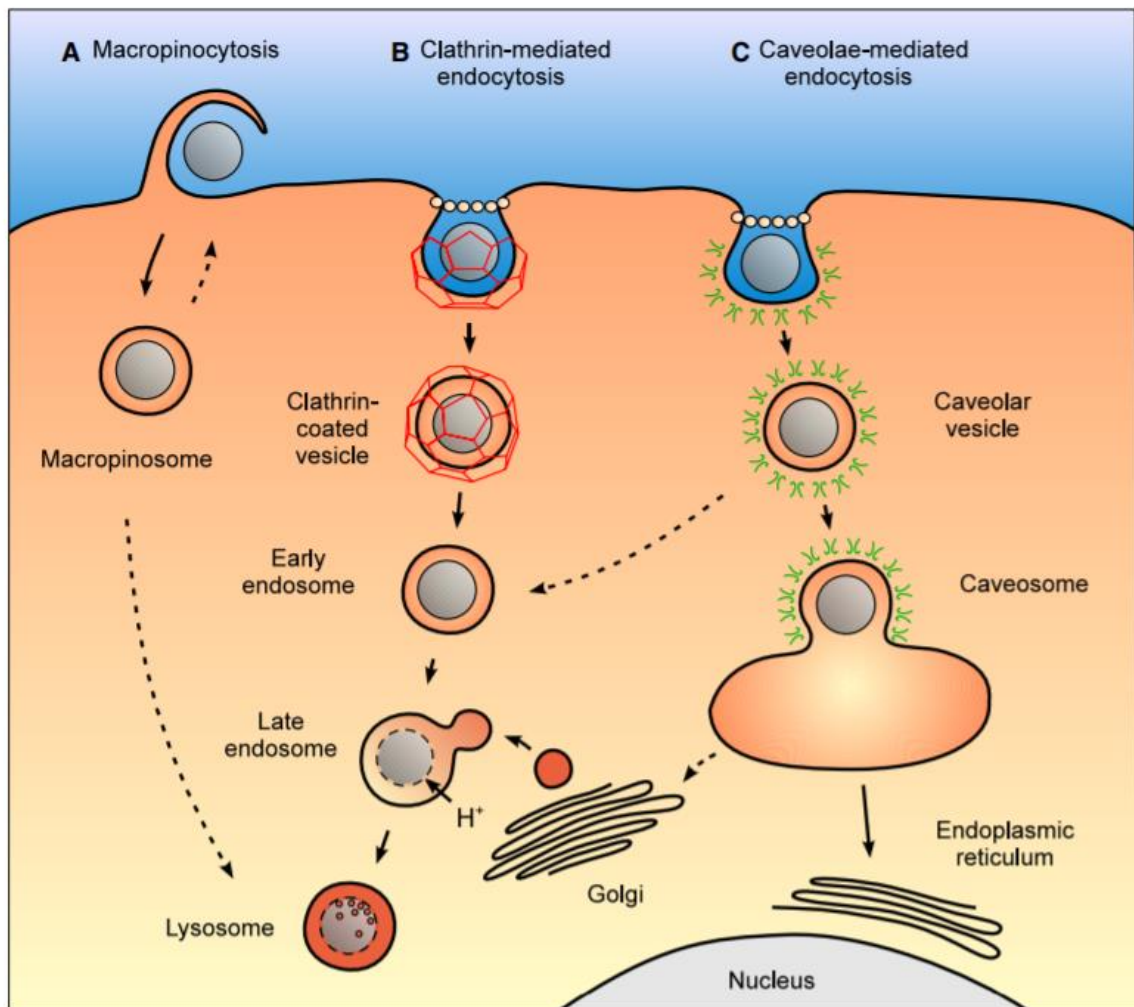


Figure 1.8. Schematic representation of caveolae dependent endocytosis [181]

Macropinocytosis

Macropinocytosis is an actin mediated membrane ruffling which was discovered by Warren Lewis in 1931 by speeding up the slow movement of rat macrophages using time-lapse microcinematography [182]. He named it as pinocytosis and it was later renamed as macropinocytosis with the discovery of clathrin mediated endocytosis and other micropinocytic mechanisms.

Macropinocytosis have distinct differences from the other forms of endocytosis. Macropinocytic vesicles, which are called macropinosomes, have no coatings and have size between 0.2-10 μm in diameter generally larger than clathrin-coated vesicles (Figure 1.9.) [183,184].

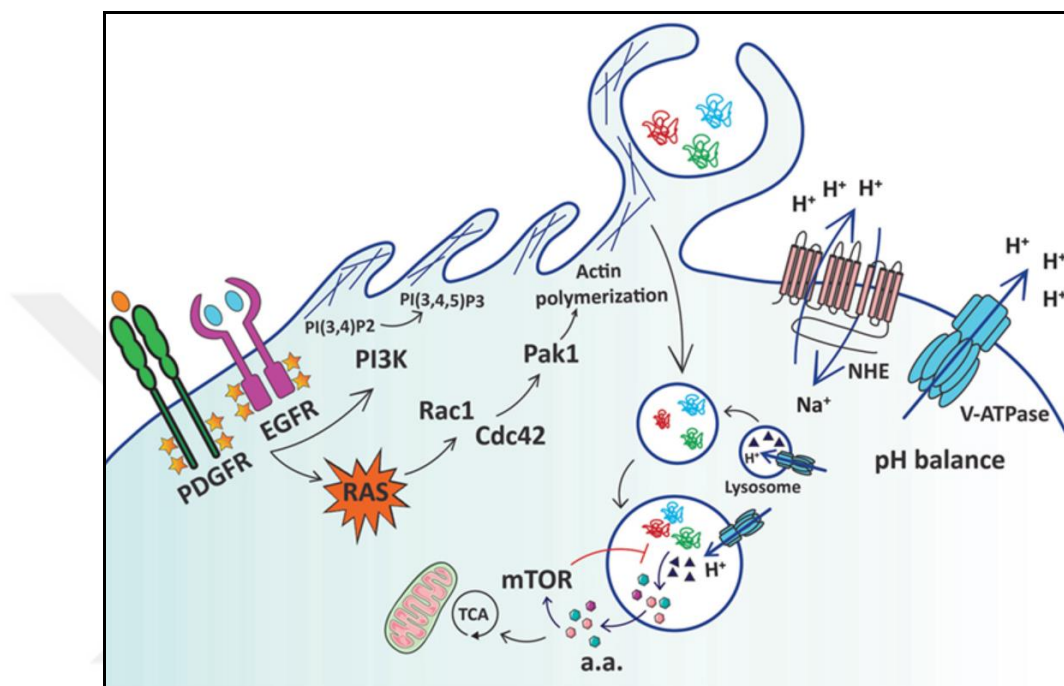


Figure 1.9. Schematic representation of macropinocytosis [185]

In the macropinocytosis, initially macropinosomes are formed from a hollow ring structure of actin polymerization. Then, this ring forms a circular ruffle and with membrane fusion; macropinosome containing external fluid is formed.

Early event in the macropinosome formation is regulated by Ras and PIP₃ [186]. Ras activates class-I PI3Ks through their Ras-binding domain, and membrane lipid enzyme PIP₃ is produced with these binding. When macropinosomes are formed from the closure of membrane, PIP₃ is lost from vesicles and give rise to PI(3,4)P₂ and then PI3P with the maturation of macropinosome [187].

In vesicle formation, site selection and vesicle coating steps; many proteins are involved which are referred as endocytic coat complex. This group contains many proteins, which perform a range of functions. Ent1p and End3p interact with PIP₂ and drive initial stages of membrane curvature. Sla1p binds endocytic cargo and other coat proteins and

negatively regulates the Arp2/3 activator Las17p and with Sla1p phosphorylation through Ark1p and Prk1p, it is released from the coat of vesicle. Sla2p binds other coat components; Pan1p binds coat proteins End3p and Yap1801/2 and activate Arp2/3. With the phosphorylation of Pan1p with Ark1p and Prk1p, it inhibits the activity of Arp2/3 dependent actin polymerization [188]. After the site selection and vesicle coating, these proteins disassemble from the vesicle. Actin involvement starts with the slow movement of detached vesicle from membrane. At this stage, with the activation of the Arp2/3 complex activation; WASP and the type I myosins start to nucleate actin filaments [189]. WASP interacts with CD2AP for the inactivation. Then, WASP recruits syndapin to allow initiation of actin nucleation at the endocytic site. WIP and type I myosins was also recruited by WASP which is used for the initiation of actin-nucleation-promotion activity [190-192].

Moreover, WASP is found on the membrane for a prolonged period but type I myosins and several regulatory proteins such as WIP/WIRE, syndapin found on the membrane just prior to actin and remain during scission and invagination as non-motile complex. Obtained filament formation starts membrane invagination and pushes the forming vesicle away from the membrane. When vesicle is bud out from the membrane, mentioned proteins are disassembled from the endocytic site [188].

After nucleation step, actin filaments are capped and bundled with the help of capping protein Cap α and Cap β which form the CapZ. Capping protein limits monomer addition and facilitate rapid filament growth [193,194].

After actin polymerization, filaments are bound together with the activity of fimbrin which is an actin-bundling protein. Obtained actin bundles become stronger than single actin filaments and provide the strength which provides sufficient force for the movement of formed vesicle. Stability of actin network is obtained with proteins such as transgelin. For the release of monomers, filaments are depolymerized with the help of cofilin [195,196].

When membrane invagination is ended, vesicle scission is started with Rvs161p and Rvs167p proteins. Amphiphysin bind to many endocytic proteins such as Las17p, Sla1, and actin [197,198]. Proteins arrive after actin then, show transient movement into the cell. These proteins have BAR domains which bind and tubulate membranes. Tubulation facilitates the scission of the vesicle. With the help of the actin bundles and coat structure;

vesicle is pinch off from the membrane with neck elongation. After scission, vesicle loses coating, move away from the membrane and fuse with endosomes (Figure 1.9.) [187].

Other Pathways

Clathrin and caveolae independent endocytosis pathways RhoA dependent endocytosis, CDC42 dependent endocytosis, ARF6 dependent endocytosis and flotilin dependent endocytosis are the pathways that are still need to be studied in detail. Their characteristic properties are shown in Table 1.2.

Table 1.2. Properties of Pinocytosis Pathways [15,34,99]

Pinocytosis Pathway (Based on proteins)	Size of Cargo	Endocytosis Proteins	Dynamin Dependency
Clathrin	<300 nm	Clathrin, AP2, epsin, actin, amphiphysin, endophilin, Rab5	+
Caveolae	<80 nm	Caveolin, cavin, actin	+
RhoA	<200 nm	RhoA, Rac1	-
CDC42	<50 nm	Cdc42, Arf1	-
ARF6	<100 nm	Arf6	Unclear
Flotilin	<100 nm	Flotilin 1 and 2	Unclear

In RhoA dependent endocytosis; RhoA is the main regulator of cytoskeleton dynamics and actin filament rearrangement for the endosomal pathway and it has important roles in also gene expression, cell-shape determination and cell proliferation [199,200]. In CDC42 dependent endocytosis, cell division cycle 42 (CDC42) protein is involved for the regulation of actin cytoskeleton and cellular stress pathways which are the key points for the control of cell growth, differentiation and apoptosis. ARF6 dependent endocytosis is regulated by ADP-ribosylation factor 6 (ARF6) which controls membrane curvature. With the control of membrane curvature, ARF6 provides membrane budding via the insertion of its BAR domain within phospholipid membrane [201]. This type of endocytosis was found as associated with tubular rather than vesicular structures [202,203]. In flotilin dependent endocytosis, flotilins are used for the induction of plasma membrane clustering for the

internalization [204,205]. Flolitins are palmitoylated lipid membrane domain markers which have a hairpin structure when bound to the plasma membrane [15].

1.2.2. Endocytosis of AuNPs

With nanotechnology idea and the ability to modify nanoparticles with desired agents, role of endocytosis in nanoparticle internalization is become crucial. With the targeted delivery of molecules to the special pathways using nanomaterials, intracellular trafficking of these molecules can be controlled. For example, when molecules are uptaken by clathrin dependent endocytosis; they are destined for lysosomal degradation; while when clathrin independent pathway is used; molecules are accumulated in endosomes and sorted to a non-degradative path.

It is known that, same type of cargo can undergo different type of internalization pathways; because different endocytosis pathways can be specifically upregulated in extent and is not always easy to distinguish different pathways from each other [32].

From another view, it is also known that uptake of the nanomaterials and the related endocytosis mechanism is affected with the size, shape, surface chemistry and the aggregation tendency of the nanomaterials [206-208].

Endocytosis of nanomaterials can be thought as a result of competition between thermodynamic driving force for wrapping and the receptor diffusion kinetics. The balance between thermodynamic driving force and receptor diffusion kinetics determines the speed and the number of nanomaterials internalized by cells. Thermodynamic driving force refers to the required free energy to take the nanomaterials to the inside of the cell and receptor diffusion kinetics refer to the kinetics of recruitment of receptors to the binding site [209]. Moreover, this phenomenon affects the behavior of nanoparticles to form clusters. It was found by Gao et al. that while 50 nm nanoparticles could enter the cell as a single nanoparticle, the nanoparticles smaller than 50 nm cannot enter individually due to the absence of enough free energy to completely wrap the nanomaterials on the surface of membrane. Thus, small nanoparticles should be clustered for the internalization. For instance, 14 nm particles should form at least six nanoparticle clusters for the uptake. It was also found that 50 nm have the fastest wrapping time with the enough energy

produced by receptor-ligand interaction to take nanomaterials into the cell. When particle become bigger than 50 nm, wrapping time of the membrane become slower due to the slower receptor diffusion kinetics [14].

For the investigation of the endocytosis mechanisms of AuNPs, Chithrani and Chan used different size and shaped transferrin coated AuNPs (14 and 50 nm gold spheres and 20x30, 14x50 and 7x42 nm rods). Transferrin is used for the restriction of the endocytosis pathways as receptor mediated ones. With the use of different inhibitors, they found that all of the nanomaterials are use receptor mediated endocytosis especially clathrin dependent endocytosis. For the uptake of the nanoparticles, they investigated that uptake of the nanomaterials are cell type and size dependent and the highest rate of uptake is obtained with 50 nm AuNPs. They also investigated the exocytosis of the nanoparticles and found that smaller nanoparticles are exocytosed faster than larger ones which is also cell-type dependent [209]. Mironava et al. investigated the uptake mechanism of different sized AuNPs and they found that 13 nm AuNPs are internalized through non-receptor mediated endocytosis such as phagocytosis while uptake of the 45 nm AuNPs is dominated by the clathrin dependent endocytosis and phagocytosis also plays role in the internalization [210]. When effect of the shape of the nanoparticles was investigated, it was found that shape affects the route of the internalization. Xie et al. used gold nano stars, rods and triangles and found that gold nano triangles uses micropinocytosis and clathrin dependent endocytosis; gold nano rods uses clathrin dependent endocytosis and cavolae dependent endocytosis and nanostars uses micropinocytosis and clathrin dependent endocytosis at the same time [211].

1.2.3. Endocytosis, Nanomaterials and Protein Corona

When explaining the interaction of NMs with the cells, it cannot be thought that they are 'bare' in their surrounding medium. Term of "protein corona" (PrC) refers the protein coating on nanomaterials which has dynamic and special structure. Formation of the protein corona is based on the high surface free energy of the NMs which is decreased by adsorption of various proteins with different binding affinities. Importance of the protein corona comes from that they represent "what the cell sees" [212]. Furthermore, protein

corona structure determines the fate of nanomaterial such as uptake, cytotoxicity, immunologic response, clearance rate, volume of distribution, organ disposition etc [213].

When NMs are encounter with the cell culture medium or blood, they will first coated with a soft corona (SC), which formed from loosely bound proteins with weak interactions and it dissociates quickly and give their place to hard corona (HC), which is formed from the proteins with stronger binding affinity and longer surface residence time [214]. Moreover, HC could be considered as the proteins directly interacting with the NM via protein-NM and protein-protein interactions while SC is formed from the proteins interacting with the inner HC via protein-protein interactions [215]. Once NMs covered with hard corona, it is recognized by cell as PrC-NMs complex and they travel through the endocytic pathway as protein PrC-NMs complex [216,217]. Moreover, it is known that SC is dissociated quickly during translocation of the PrC-NMs complex while HC stay still on the NMs surface until they are degraded in the lysosomes [217,218]. Due to removal of soft corona during cellular route of the NM, hard corona is thought to be has a greater role determining physiological response of NMs [219]. On the other hand; it could also be though that SC could have important role to interaction of NMs with living systems such as defining circulation stability of the NMs [215].

Determination of the HC and SC is not based on well-known and well-established experimental setups and protocols. For the determination of protein quantification, protein affinity, conformation, interaction and identification of proteins, several methods were developed. For the separation of PrC, mainly centrifugation [220], size exclusion chromatography [221] or magnetic separation was preferred [222]. PrC structure could be analyzed by dynamic light scattering (DLS) [223], differential centrifugal sedimentation (DCS) [212], Quantification is generally based on colorimetric assays such as Bicinchoninic acid (BCA) assay and Bradford assay [224] besides thermogravimetric analysis [225] is also used. Protein binding affinity, stoichiometry and interaction are analyzed generally by fluorescence correlation spectroscopy (FCS) [226], isothermal titration calorimetry (ITC) [221], surface plasmon resonance (SPR) [227]. Protein conformation is examined by circular dichroism (CD) [228], Fourier-transform infrared (FTIR) spectroscopy [229], Raman spectroscopy [229], nuclear magnetic resonance (NMR) spectroscopy [230], and differential scanning calorimetry (DSC) [231]. Composition of the PrC could be investigated by gel electrophoresis [232] and mass

spectroscopy [233]. All of the mentioned techniques are appropriate for the examination of HC properties despite most of the techniques are insufficient to work with SC. Because separation of the SC from the environment is more difficult than separation of HC, HC can be easily separated from surrounding environment by centrifugation and the remaining analysis could be done to analyze HC structure. However, the most of the separation methods for SC stress PrC-NMs complex and cause partial or total detachment of the SC from the complex. Thus, there are limited number of methods for the isolation and determination of SC. Studies are generally based on *in situ* measurements which include investigation the SC directly in the protein solution and *ex situ* measurements which includes investigation the SC after isolation which is not a common method for SC analysis. Both of the techniques could only give an approximation regarding the SC structure [215]. Thus, novel methods should be developed for especially SC analysis which gives vital information for the interaction of NMs with surrounding environments.

Structure of the protein corona is determined by NMs properties including size [234], hydrophobicity [221], surface chemistry [235] and charge [236], surface roughness [237], curvature [238] and shape [239], and also protein identity in the surrounding environment [221]. For the AuNPs-PrC identification; many studies have been reported. When PrC of AuNPs were investigated; various proteins were found to adsorb on the AuNPs surface such as ubiquitin [240], serum albumin [241], tumor necrosis factor [242], cytochrome C [243], fibrinogen [244], or polypeptides [245]. Moreover, when effect of the size of AuNPs on the PrC formation was examined, it was found that size affects the adsorbed number of proteins on the AuNPs surface because size changes the curvature of the NPs which have different protein binding constants. Moreover Deng et al. found that fibrinogen binding affinity to AuNPs increases with increasing size of AuNPs [244]. Lacerda et al. also showed that when size of the AuNPs increase; their capability of binding plasma proteins also increase and also when AuNPs size increases, adsorbed proteins undergo conformational changes and adsorbed protein layer increases [246]. Not only size but also shape affects PrC formation on AuNPs. It was showed that PrC formation on gold nanorods (AuNRs) and gold nanostars (AuNSs) changes and amount of adsorbed protein on AuNSs were found as larger than those on AuNRs. 70 nm AuNRs and AuNSs cause larger number of protein adsorption when compared with 40 nm AuNRs and AuNSs although 40 nm AuNSs adsorbed lower amount of protein but higher number of identified

proteins when compared with 70 nm AuNSs [247]. Furthermore, Cui et al showed that modifications on the AuNPs surface including modifications by citrate, thioglycolic acid, cysteine, PEG could alter PrC structure and PEG coating can decrease the adsorption on the AuNPs [248]. Vonarbourg et al. also showed that citric and phosphine modified AuNPs adsorbed more proteins while polymer coated AuNPs including PEG, poly (isobutylene-alt-maleic anhydride) adsorbed less proteins and explained that large steric hindrance of the polymer coating on the AuNPs could inhibit protein adsorption on AuNPs when compared with small molecules. Similar with Cui et al they also showed that PEG coating reduced protein adsorption [249].

1.2.4. Role of the Endocytosis in the Immune System and Macrophage Activation

Endocytosis is not only used for the internalization of molecules from surrounding environment but also used for the regulation of microbial infection, clearance of pathogens, antigen presentation process and also receptor signaling [250,251].

Neutrophils, dendritic cells (DCs) and macrophages are cells which drive innate immune response as phagocytes. Neutrophils are the first defense mechanism against infectious diseases [252] and express Fc- and complement-receptors which used for the phagocytic movement and kill ingested bacteria [253]. They are also used for the activation and recruitment of macrophages to the infectious site and activation of dendritic cells (DCs) [254,255].

DCs are professional antigen-presenting cells which present antigen by MHCII molecules to CD4⁺ T cells. DCs use fluid phase macropinocytosis or receptor mediated endocytosis for the internalization of antigens for the presentation on MHCII molecules. On the other hand, DC maturation is also related with the macropinocytosis where maturation of DC requires increased uptake of antigens associated with inflammatory stimuli [256].

Macrophages play a vital role in tissue homeostasis and inflammation with the clearance of pathogens and dead cells. Inflammation is initiated by macrophages principally through phagocytosis, release of cytokines, formation of reactive oxygen species and concluded with the activation of immune system [257].

Macrophages have a plasticity which provides them to alter their activation phenotype into classical (M1) or alternative (M2) [258]. The activation of macrophages is essential for the trigger of the immunity which is initiated by the recognition of stimuli and phagocytosis [259]. When macrophages are activated with any type of stimulation; their morphology, metabolism and function changes [260,261]. There are 2 activated type of macrophage phenotypes as classically activated (M1) and alternatively activated phenotype (M2) (Figure 1.10) [261]. T cells provide polarization/activation of these two types of macrophages. Lipopolysaccharide (LPS), interleukin-2 (IL-2), gamma-interferon-gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α) induce M1 phenotype of macrophage. These macrophages release proinflammatory cytokines such as TNF- α , IL-1, IL-6, IL-12, and IL-23 [262-264]. Production of these cytokines provides development and expansion of helper T cells (Figure 1.10.a). M1 phenotypes express highly MHC-1 complementary factors and Class II antigens for phagocytosis and they promote arginine metabolism into nitric oxide and vitruiline by secreting inducible nitric oxide synthase (iNOS) [261,265] (Table 1.3.). With those functions, M1 macrophages play a vital role in host defense for the protection from various bacteria, protozoal or viral infections with the generation of reactive oxygen and nitrogen species [266]. iNOS enzyme produces NO with the use of L-arginine due to its anti-bacterial and anti fungal properties [267-270].

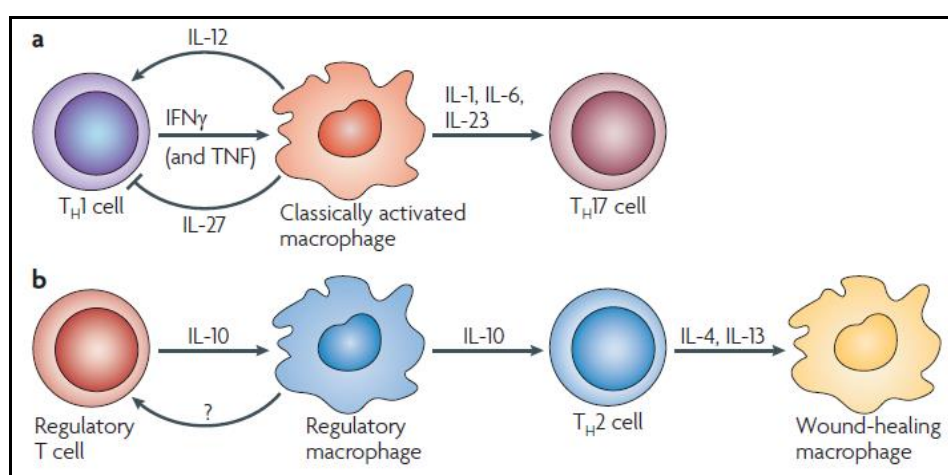


Figure 1.10. Type of alternatively activated macrophages and their interactions with T cells [271]

With the damage response; secretion of immunocomplexes, glucocorticoids, anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 induce M2 phenotype of

macrophages [272,273]. They express IL-10, mannose receptor and scavenger receptor. The major functions of M2 phenotype macrophages are suppression/regulation of inflammatory responses, regulation of wound healing, immune modulation, tissue remodeling, parasite clearance, and tumor progression [261,274,275]. M2 phenotype can be subdivided into M2a, M2b and M2c phenotypes according to their functions.

M2a, also known as wound healing macrophages (Figure 1.10.b), is activated by IL-4 or IL-13 which provides the conversion of macrophages into a population of cells that are programmed to promote wound healing. IL-4 stimulates arginase activity which allows conversion of arginine to polyamines and collagen precursor ornithine. This conversion provides production of the extracellular matrix in wounded tissue [276]. M2b is activated by immune complexes in combination of IL-1 β or LPS. It has role in activation of T-helper cells, B-cell activation and also wound healing with the stimulation of proliferation of fibroblasts [277]. M2c, also known as regulatory macrophages (Figure 1.10.b), is activated by IL-10, transforming growth factor (TGF- β) or glucocorticoids. Glucocorticoids are released in response to stress and can have a role in macrophage mediated defense mechanism and inflammatory response [278]. Production of TGF- β provides phagocytosis of apoptotic cells in the presence of pro-inflammatory stimuli [202,279].

Table 1.3. Properties of Activated Macrophages [261,271]

	Cell Type			
	M1	M2a	M2b	M2c
Stimulatory Factors	IFN- γ , LPS, TNF- α	IL-4, IL-13, glucocorticoids	TLR/I L-1R ligands	IL-10
Cytokines Secreted	TNF- α , IL-1 β , IL-12, IL-23	IL-10, TGF- β , IL-1Ra	TNF- α , IL-1, IL-6, IL-10	IL-10, TGF- β
Chemokines and Receptors	CCL5, CXCL9, CXCL10, CXCL11, CXCL16, CXCL3L1	CCL17, CCL18, CCL22, CCL24	CCL1	CCL13, CCL16, CCL18
iNOS	+	-	-	-
Cell Surface Markers	CD80, CD86, TLR2, TLR4, ILIRI	CD163, CD206, ILIRII	CD86	CD163, TLR1, TLR8
Receptors	Mannose Receptor	Mannose and Scavenger Receptor	-	-
Killer Molecules	NO, O ₂ ⁻	-	-	-

When nanomaterial interaction with the immune system is considered; nanomaterials can activate innate immune response, complement system and adaptive immune response [280-282]. Although most of the nanomaterials cause activation on the immune system; they can also suppress immune response [282]. For the effect on the immune system; different physicochemical properties of the nanomaterials play important role such as size, distribution, aggregation, surface modifications and composition, crystallinity [283]. Besides the physicochemical properties of the nanomaterials; biological properties should also be taken in to account including impurities of endotoxins from the synthesis [284], biological environment behaviors of nanomaterials involving changed size or surface properties due to the interaction with cell culture media or body fluids [285].

For AuNPs, as a first study Shukla et al., investigated the interaction of 3 nm AuNPs with macrophages in detail in 2005 and found that small AuNPs used pinocytosis for internalization and localized mostly in lysosomes and perinuclear space. They also showed that AuNPs suppress the production of reactive oxygen species and do not cause any change in the proinflammatory cytokines production [286]. On the other hand, another report showed that AuNPs with three different sizes as 3, 5 and 40 nm can cause reduction of the macrophage number and increase the size of the cells with increased expression of proinflammatory genes IL-1, IL-6 and TNF- α as size dependent [287]. Sumbayev et al. used 4 different sized AuNPs (5, 15, 20, and 35 nm) and interacted them with THP-1 human leukemia monocytic macrophages and showed that AuNPs down regulate IL-1 β *in vitro* and also *in vivo* as size dependent manner [288]. AuNPs were also interacted with natural killer (NK) cells, dendritic cells (DCs) and lymphocytes and found that 12 nm AuNPs can affect DCs maturation, T helper lymphocyte proliferation, and also proliferation of inflammatory NK cells and proinflammatory cytokine secretion while 2 nm AuNPs has higher uptake rate without any effect on dendritic cell maturation or lymphocyte proliferation [289]. Besides the bare AuNPs, also interactions of the modified AuNPs with immune system were also investigated. Bastus et al showed that bare 10 nm AuNPs do not induce production of proinflammatory cytokines while peptide modified AuNPs cause induction of NO synthase and proinflammatory cytokines TNF- α , IL-1, IL-1 β and IL-6 with inhibited macrophage proliferation [290,291]. Moreover, Lee et al. found that both bare Au nanorods (AuNRs) with averaged aspect ratio 2.6 and SiO₂-coated AuNRs cause inflammatory mediator production such as cytokines, prostoglaains with

activated immune response genes [292]. Cytosine–phosphate–guanosine (CpG) oligodeoxynucleotides were also used for the conjugation with AuNPs which provide activation of the macrophages via the interaction with TLR9 receptors. With the conjugation, uptake of the AuNPs can be enhanced and proinflammatory cytokine release can be increased [293].

1.3. RAMAN SPECTROSCOPY

When the energy of an incident photon matches with the energy gap between the ground state and an excited state of a molecule, absorbed photon can provide the excitation to the higher energy state and when photon emission occurred at a longer wavelength, this is called fluorescence. On the other hand, if the incident photon cause excitation to a virtual energy state and inelastic scattering, this is called in general as Raman scattering [294].

Single frequency of radiation is used in Raman spectroscopy to irradiate a sample and vibrational energy different from the incident beam is detected. Thus, Raman scattering does not need matching of the incident radiation to the energy difference between the ground and excited stated. In Raman scattering, when the light interacts with the molecule and cause polarization, the cloud of electrons round the nuclei to form a short-lived state called a virtual state. The energy of these states is determined by the frequency of the light source. Then, the photon is quickly re-radiated [294].

If only electron cloud distortion is involved in scattering, the photons will be scattered with small frequency changes, as the electrons are comparatively light. This scattering process is called Rayleigh scattering and regarded as elastic scattering and is the dominant process. It does not involve any energy change; thus, light returns to the same energy state.

If nuclear motion is induced during the scattering process, energy will be transferred either from the incident photon to the molecule or from the molecule to the scattered photon. These inelastic scattering processes are called as Raman scattering and the energy of the scattered photon is different from the incident photon. Raman scattering is weak process that only one in every 10^6 - 10^8 scattered photons is Raman scattered [294] and if the scattered light loose energy, the scattering is called Stokes scattering while if the scattered light gains energy due to being in an excited state with thermal energy, it is called anti-

Stokes scattering. (Figure 1.11.). Relative intensities of anti-Stokes and Stokes processes depend on the population of the various states which are determined by the Boltzman distribution of the molecule and at room temperature, the number of molecules in the excited state is smaller than the ones in the low-energy states. Because of this reason, intensity of the anti-Stokes scattering is weaker than Stokes scattering and intensity decreases with the frequency of the vibration increases due to the decreased population of the excited vibrational state [294,295].

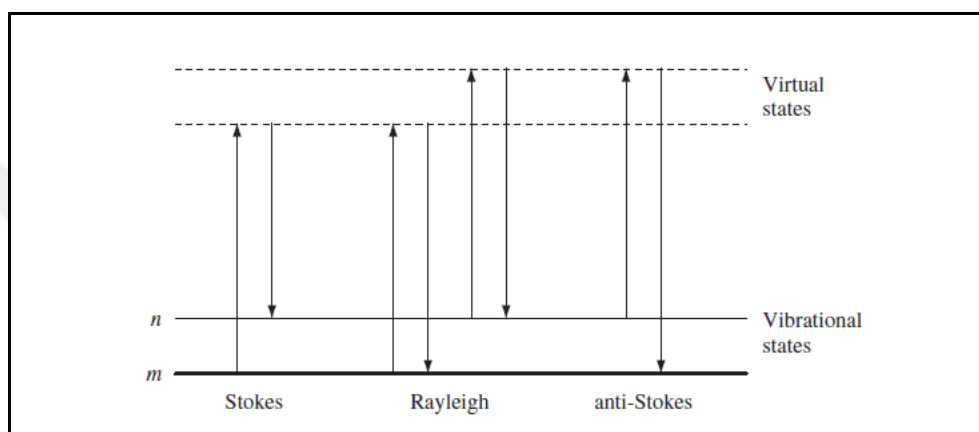


Figure 1.11. Diagram of Rayleigh and Raman scattering processes [294]

Detected energy changes are coming from nuclear motion in vibrational spectroscopy. In scattering process, change in the energy of photons cause a change in the frequency which is resulted as shift in frequency relative to the excitation photons by the energy of characteristic molecular vibrations [296]. Because of this reason, Raman spectrum has several different "Raman lines" generated by different molecular vibration scattering which provides a vibrational "fingerprint" of a molecule.

The Raman spectroscopy is promising vibrational technique for the analysis of biological substances due to the obtained valuable information coming from the chemical structure of the probed substances [297,298]. Raman spectroscopy was used for the analysis of living cells within the recent decade. With the confocal Raman systems cell nucleus and cell cytoplasm was differentiated [299-303]. Despite Raman spectroscopy is a promising tool for biological molecules; it is not sensitive enough for the detection of molecules in the cell. Biomolecules are found in cells at very low concentration and excitation laser intensity of is restricted due to the cellular damage, thus, obtained Raman signal is become

extremely weak. Besides, almost all biomacromolecules are weak Raman activity. In SERS, Raman signals can be enhanced with the use of metallic nanostructures [304].

1.3.1. Surface-Enhanced Raman Spectroscopy

Surface-enhanced Raman spectroscopy (SERS) was first observed in 1974 and verified in 1977, when spontaneous Raman signal of adsorbed pyridine was easily measured at a roughened silver electrode [305-307].

When an electromagnetic wave interacts with a noble metal surface, the localized surface plasmons provide an amplification of the electromagnetic fields produced by conduction electrons at material surface that undergo collective oscillations. There are two enhancement mechanisms which are resulted in enhancement by factors of 10^6 - 10^{10} in SERS [308]. First enhancement is called as electromagnetic enhancement and it is related with the resonances between the surface plasmons of the metal nanostructures and scattered fields during Raman process which provides the enhanced local excitation fields. This resonance causes a redistribution of field intensities in the vicinity of nanoparticles resulting in areas of enhanced excitation intensities for the Raman process. Additionally, based on the same resonance mechanism, nanostructures can be considered as nanoantennas for transmitting and enhancing Raman scattering. Electromagnetic enhancement depends on the resonance between plasmons and excitation and scattered fields, where plasmon frequencies are determined by the material and the morphology of the metal nanostructures [296]. Second enhancement is also called as charge transfer or chemical enhancement. In this enhancement, a bond is formed between the analyte and the metal surface. This bond produces surface species formed from both analyte and surface metal atoms. With formed bond, charge transfer is become possible from the metal surface into the analyte. The enhancement is occurred with raised new electronic state from the formation of new bond [294].

Recently, for the intracellular processes and related biochemical changes in single cell level, many biochemical methods such as microarrays, PCR, etc. are used. Disadvantage of the currently used methods is the disruption of the cellular integrity. With the use of these methods, it is not possible to work *in situ* because they require fixation, staining or lysis of the cells [309]. Moreover, most of the used methods require large number of cells, long

sample preparation times and higher costs for the analysis [310]. Due to these disadvantages of the current methods; there is a need for the single live cell analysis which can provide the analysis of the real-time intracellular processes. As an alternative and noninvasive method, SERS is a powerful technique for the single cell analysis which provides label-free detection of molecules from living systems.

SERS can help to obtain detailed information on molecular structures in the nanometer-scaled environment of the nanoparticles [297,311]. As a nanometer-scaled environment; investigation of intracellular structures is important to study with SERS which can be able to detect nanomolar concentrations with the use of metallic nanomaterials. It provides spectral details without interference coming from the vibrational peaks of H₂O which causes interference in IR spectroscopy. Moreover, it gives opportunity to work non-destructive and label-free with simple or no preparation of samples [308,312].

As SERS substrates, in the intracellular studies, gold and silver nanoparticles are the most preferred substrates [168,296,304,311,313-320]. Especially, gold nanoparticles are preferred due to their favorable physical and chemical properties for the living cell analysis. Their high electron density can be used as an advantage for labeling in electron microscopy [311,321-323]. They can be easily synthesized and their surface properties can be also easily tailored. For the interaction with cells and biological molecules, their biocompatibility and chemical stability also play an important role. Since they have many different advantages, most important one is their stronger enhancement of Raman signals for SERS studies. Spherical gold nanoparticles (AuNPs) with the size between 20-60 nm exhibit the best SERS enhancement factors of 10^{14} and provide Raman measurement of single molecules without any clustering [304,308,311,324].

For intracellular studies, enhancement mechanism depends on many factors such as type, morphology, size, shape, and surface chemistry, aggregation of nanomaterial and interaction of nanomaterial with cells and also uptake and toxicity of nanomaterial. For intracellular studies, particle diameter must be small enough to internalize and larger enough (larger than 15 nm) to achieve SERS enhancement [308,311].

In intracellular SERS studies, gold nanoparticles can be used for two different purposes: (i) they can be used as SERS labels which are conjugated to the reporter molecules such as dyes and this reporter molecule can be detected by their specific Raman signature. (ii) they

can be used as nanosensors which can deliver enhanced Raman signature of biological molecules and structures in the environment [296,314,315,325-331]. These two purposes can also be combined and SERS signature of receptor molecule with the enhanced Raman signature of the environment can be obtained simultaneously [311].

In the very first studies, SERS was used the investigation of the intracellular distribution of drugs and antitumor drugs/nucleic acid complexes in living single cells but there was not any information about the SERS spectra of native cells [332-334].

In 2002, Kneipp et al, obtained SERS spectra of living single cells with the use of 60 nm colloidal AuNPs [304]. Then, the same group investigated the spectral changes in the living cells with time [311]. They used 30-50 nm AuNPs which have high uptaken rate and used pulse-chase method which is based on the incubation of the cells with nanoparticles for a certain time then incubation of the cells without any nanoparticles. With this method; in the chase phase there cannot be any nanoparticle internalization and all endosomes contains approximately the same amount of AuNPs. They saw that vibrational modes of different cellular molecules such as proteins, carbohydrates, lipids, and nucleotides in endosomal compartments can be detected via SERS and time dependent SERS spectra changes is based on the both aggregation levels of AuNPs and the maturation of endosomes [311]. In their other study, they used 14 nm AuNPs to obtain intracellular SERS spectra from fibroblast cells. They obtained an increase in the number of the SERS spectra per cell when they increased the analysis time (chase time) with 30-minute constant AuNPs incubation. This result is explained with the formation of the AuNP aggregates during endosomal maturation, multivesicular fusion and their effect on the nanoparticle stability during the endosomal trafficking. They found that after 48h, number of the SERS spectra is decreased which can be explained with the exocytosis of the AuNPs. Moreover they found that when incubation time of the cells with AuNPs is changed, obtained spectra also changed which means the composition of the endosomal surroundings and the interactions between the biomolecules in the endosomes and AuNPs changes [335].

Fujita et al. was also investigated time dependent spectral changes after the endocytosis of AuNPs [336] and with the combination of dark-field microscope with a slit-scanning Raman microscope, they tracked movement of AuNPs and related spectral changes with that movements [337,338]. In their later reports, with the use of 50 nm AuNPs and same

system; they have revealed that obtained SERS spectra should be originated from the molecules working for cellular transportation such as kinesin or dynein [339,340].

Kneipp et al. also determined pH changes during the endocytosis of 80-120 nm gold and silver nanoaggregates with the use of 4-mercaptobenzoic acid (pMBA) [341,342] and also with 30-50 nm AuNPs and two reporter Rose Bengal and Crystal Violet [315] and in these two reports they found that maturation of endosomes starting from early endosomes to late endosomes/endolysosomes can be detected using pH reporter SERS nanosensors. Same group also used 5 different reporter molecules (pMBA, para-aminobenzenethiol (pABT), rhodamine B isothiocyanate (RBITC), crystal violet (CV), 2-naphthalenethiol (2NAT)) to gather structural information from intracellular environment by using special statistical methods such as principle component analysis, hierarchical cluster analysis, and K-means clustering analysis [319]. pMBA was also used by Sirimuthu et al for the investigation of redistribution of AgNPs during cell division [343]. Campbell's group was used not only pMBA but also 1,8-diaza-4,5-dithia-1,8-di(2-chloro-(1,4)-naphthoquinone-3-yl)octane (NQ) and 2-Mercapto-benzene-1,4-diol (HQ) for the measurement of intracellular redox potential and pH measurements in living cells with the use of gold nanoshells [344-348]. Surface-enhanced hyper Raman scattering (SEHRS) was also used for the investigation of intracellular processes with the use of Raman reporters pMBA and 2NAT [349].

Lysosomal degradation was also investigated with SERS by using AuNPs as substrates. Lucas et al used healthy and cancerous EGFR overexpressing cells and EGFR tagged 30 nm AuNPs for the investigation of degradation during endocytosis. They have found that obtained SERS spectra differed in different locations inside and outside of the cell which is collected by mapping with 0.3 μ m. Altered SERS spectra explained as the places where the lysozyme degrades the protein [350].

Cell based differentiation was also achieved by SERS. Huang et al. used 4 different sized AuNPs as 27, 42, 55, and 70 nm for the differentiation of human hepatoma carcinoma and human hepatic cell line. They have investigated SERS spectra on different time points such as 0.5, 1, 3, 6, 12, and 24 h and found that 55 nm AuNPs with 6 h incubation time gives better spectrum [351]. Neuroblastoma cell differentiation was also investigated by Huefner et al. They used 40 and 60 nm bare AuNPs, fictionalized AuNPs with NLS peptide and found that for bare AuNPs, 60 nm AuNPs are internalized more than 40 nm AuNPs. They

obtained spectral differences coming from the metabolic changes induced by differentiation and they discriminated the cellular SERS spectra by PCA and LDA [352]. Kuku et al. differentiated three types of cells, which were diagnose metastatic, primary and non-cancerous cell lines via SERS with the help of PCA and LDA. On the other hand, in the same study, they have also investigated the cellular response against different type of nanomaterial such as zinc oxide nanoparticles (ZnO NPs), titanium dioxide nanoparticles (TiO₂) and single-walled carbon nanotubes (SWCNTs). Then in another study, they have investigated the toxicity of nanomaterials without any reporter molecules. They found that with the increased number of different nanomaterials; obtained SERS spectra can vary. The changes can be correlated with the cellular viability using cytotoxicity and apoptosis-necrosis assays [353]. With the use of two different reporter molecules, ((1,8-diaza-4,5-dithia-1,8-di(2-chloro-{1,4}-naphthoquinone-3-yl)octane and para-mercapto benzoic acid) the overall redox potential and the pH of cells were detected with SERS. Obtained redox potential is used for the deduction of the reactive oxygen and nitrogen species formation which can be thought as a result of toxicity [346]. Reactive oxygen species induced DNA damage using silver nanoparticles [354] and the hydrogen peroxide induced apoptosis of the living single cells using AuNPs [355] were also investigated by the SERS.

Organelle based changes in the cells were also investigated with SERS via specific targeting. With the nuclear targeting, cell cycle of the cancer cells were also monitored via SERS with the use of AuNPs conjugated with RGD and nuclear targeting ligand peptides [356]. Same AuNPs are also used for the monitoring nuclear drug delivery dynamics in the single cells. For this purpose, doxorubicin is used as a model drug and conjugated on the AuNPs via a pH-sensitive linkage [357]. Moreover, mitosis of healthy and cancer cells [358], hyperoxia induced intracellular acidification [359], ultraviolet radiation-induced cell responses and intracellular biomolecular dynamics [360] were investigated with the help of polyethylene glycol (PEG), Arginylglycylaspartic acid (RGD) peptide and nuclear localization signal (NLS) peptide functionalized gold nanocubes. Kang et al. used mitochondria, nucleus and cytoplasm targeted AuNPs to investigate cell death induced biochemical changes [361].

2. OBJECTIVES OF THE STUDY

In this study, it is aimed to investigate whether SERS can detect the endocytosis route in living single cells. While answering these questions, it is critical to realize that the SERS spectra originate from AuNPs and their aggregates inside living cells. As AuNPs are added into culture medium, they are readily coated by cell culture proteins to form protein corona mostly determining the intracellular fate of NMs through uptake, route and clearance. Thus, first the types of the protein corona on AuNPs surfaces were investigated as they are added into the studied cell culture media. This was done to answer the question of whether protein corona contribute to the SERS spectra and provide information about the protein corona type formed on the AuNPs surfaces. The question was answered by forming different protein corona conformation in different type of cell culture conditions. Then, AuNPs uptake route was attempted to track with SERS by employing specific endosomal pathway inhibitors. Further, to investigate the source of the bands observed on the cellular SERS spectra, the endosomes containing AuNPs were isolated from the cells and their SERS spectra were obtained to compare with the SERS spectra acquired from whole cell. Finally, another mechanism, activation of macrophages was investigated to answer whether the metabolism inside of a cell, not directly related to the endocytosis, could be determined from the SERS spectra. The phagocytosis of AuNPs by activated macrophages was also examined by SERS at increasing concentrations of AuNPs in order to understand whether the SERS spectra could track not only endocytosis but also phagocytosis of activated macrophages.

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Chemicals

Gold (III) chloride trihydrate (≥ 99.9 percent trace metals basis), sucrose (Scr), chlorpromazine (Cpm), nystatin (Nys), filipin (Fil), amiloride (Aml), dynasore (Dyn), cytochalasin D (CytoD), NaN_3 , deoxy-D-glucose (dG), Percoll, imidazole, EDTA, glycine, Trizma-Base, Trizma-hydrochloride, HCl, NaOH, nitric Acid, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD), sodium nitrite, lipopolisaccharide (LPS) were purchased from Sigma-Aldrich (Darmstadt, Germany). Tri-sodium citrate (powder) was purchased from Merck Millipore (Germany). RIPA Buffer and Laemmli Buffer (4x) were purchased from Bio-Rad.

3.1.2. Cell Lines

A549 (human caucasian lung carcinoma), Beas-2b (human bronchial epithelial cell), HeLa (cervical cancer epithelium), RAW 264.7 (murine macrophage cell) cell lines were purchased from the American Type Culture Collection (ATCC, USA) and PNT1A (normal human prostate cell) cell line were purchased from Sigma-Aldrich (#95012614, USA).

3.1.3. Cell Culture Reagents

Dulbecco's Modified Eagle Medium (1x DMEM, 4500 mg/L Glucose, L-glutamine, sodium pyruvate, Phenol red), Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, 4500 mg/L Glucose, L-glutamine, sodium pyruvate, Phenol red), fetal bovine serum (FBS), Penicillin and Streptomycin (10,000 Units/ml), L-glutamine (200 mM), 1x Trypsin-EDTA (0.25 percent Trypsin-0.02 percent EDTA) and phosphate buffered saline (10X PBS) were purchased from Gibco (USA). Dimethyl sulfoxide (DMSO) (anhydrous, ≥ 99.9 percent) were purchased from Sigma-Aldrich (USA). Mouse

anti-Rab7 primary antibody, rabbit anti-Rab11 primary antibody, rabbit anti-LAMP1 primary antibody, rabbit anti-EEA1 primary antibody were purchased from Abcam (UK). Tissue culture flasks, well plates, falcon tubes, serological pipettes and cryotubes were purchased from TPP (Switzerland).

3.1.4. Kits

WST-8 cell proliferation reagent (Abcam), ATP Assay (Abcam ab83355), Cellular Reactive Oxygen Species (ROS) Assay Kit was purchased from Abcam (UK). Annexin V-FITC-/PI apoptosis necrosis detection kit was purchased from Calbiochem (Germany). The bicinchoninic acid assay (BCA) protein assay was purchased from Thermo-Fischer (USA)

3.1.5. Instruments

UV/Vis spectrometer (PerkinElmer, USA), Zetasizer Nano-ZS (Malvern Instruments, UK), benchtop pH/mV meter (Hanna Instruments HI 2211, USA), mini-shaker (Multi Bio 3D Biosan, Latvia), 2-5 centrifuge (Sigma, UK), Gel Electrophoresis System (Bio-Rad, Germany), mikro 22R centrifuge (Hettich, UK), Biohazard safety cabinet (Esco Class II type A2, USA), CO₂ incubator (37°C, CO₂ 5 percent, Nuaire, USA), ELx800 Absorbance Reader (Biotek, USA), Flow Cytometer (Guava easy-Cyte5, Merck Millipore, Germany), Raman microscopy system (InVia Reflex, Renishaw, UK) were used in this study.

3.2. METHODS

3.2.1. Synthesis and Characterization of Gold Nanoparticles (AuNPs)

AuNPs were synthesized by citrate reducing method [362]. Briefly, 10 mg of gold (III) chloride trihydrate (HAuCl₄·3H₂O) was dissolved in 100 mL of ddH₂O and solution was boiled. Then, 1 mL of 1 percent sodium citrate (Na₃C₆H₅O₇) was added into the boiling solution quickly. After sodium citrate addition, resulting mixture was boiled for 15 min and left at room temperature for cooling.

Synthesized AuNPs were characterized using dynamic light scattering (DLS) and UV/Vis spectrometer.

Protein corona formation of the AuNPs surface was characterized by DLS, UV/Vis spectrometer and SDS-PAGE. For the determination of PrC, two different cell culture media (DMEM and DMEM:F12 Ham mixture at 1:1 ratio (DF12)) and 2 different FBS concentrations (5 percent and 10 percent) were used and PrC structure was determined as soft corona (SC) and hard corona (HC). For the determination of SC, measurements were obtained *in situ* which means AuNPs did not separated from the surrounding environment and measurements were obtained in the cell culture media. However, for the characterization of HC, AuNPs were separated from both SC and environment by centrifugation. For this purpose, AuNP including cell culture media were centrifuged at 14,000 rpm for 10 min. Pelleted AuNPs were washed twice and measurements were obtained from colloidal suspension of washed AuNPs.

3.2.2. Cell Culture

Human lung bronchial epithelium (Beas-2b) cell line, human lung cancer epithelium (A549) cell line, and human normal prostate epithelium (PNT1A) cell line, cervical cancer epithelium (HeLa) cell line and macrophage cell line (RAW 264.7) were purchased from American Type Culture Collection. Beas-2b cell line was cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 percent fetal bovine serum (FBS), 1 percent Penicillin-Streptomycin (PS). A549 cell line was cultured in DMEM-F12 supplemented with 10 percent FBS, 1 percent PS, and 2mM L-glutamine. HeLa, PNT1A and RAW 264.7 cell lines were cultured in high glucose DMEM supplemented with 10 percent FBS, 1 percent PS. Cells were cultured at 37°C under 5 percent CO₂ humidified atmosphere.

3.2.3. Inhibition Studies

For the endocytosis inhibition, sodium azide (NaN_3), deoxy-D-glucose (dG), sucrose (Scr), chlorpromazine (Cpm), filipin (Fil), nystatin (Nys), cytochalasin D (CytoD), amiloride (Aml), and dynasore (Dyn) were used. For the inhibition of receptor mediated endocytosis, 10-100 mM NaN_3 [209, 363] and 10-100 mM dG [209, 363], for macropinocytosis, 10-100 μM Aml [25], and 1-10 μM CytoD [364], for clathrin mediated endocytosis, 200-500 mM Scr [209, 363] and 10-100 mM CPM [365], for caveolae mediated endocytosis, 0.1-1 μM Fil [365] and 0.1-0.4 μM Nys [363, 365], for dynamin mediated endocytosis, 10-80 nM Dyn [365, 366] were used according to the literature. For endocytosis inhibition studies after optimization, cell lines were preincubated with inhibitors for 2 h.

3.2.4. Cell Proliferation Assay

For the investigation of cytotoxicity of inhibitors, cell proliferation was measured by WST-8 assay. Beas-2b, A549, HeLa and PNT1A cells were seeded on each well of a 96-well plate with 15,000 cells/well density in triplicate. Cells were incubated for 24 h for attachment at 37°C under 5 percent CO_2 humidified atmosphere. Then, cells were exposed to inhibitors 2 h with 4 h different concentrations. After 2 h treatment of inhibitors, inhibitor containing media was removed and fresh media was added to mimic flow cytometry and SERS experiments. For the cytotoxicity assessment of AuNPs, cells were treated with 1.6×10^{15} AuNPs as determined in previous studies [353, 367] without inhibitor treatment.

For the cytotoxicity of AuNPs on the RAW 264.7 cells, cells were seeded on each well of 96-well plate with 100,000 cells/well density in triplicate to mimic the LPS activation studies. Cells were incubated 24 h for attachment at 37°C under 5 percent CO_2 humidified atmosphere. Then, cells were exposed to 25 percent (v/v) AuNPs for 24 h without any LPS treatment (Only Au).

After total 24 h incubations, cells were washed with phosphate buffered saline (PBS) once and incubated with 5 percent v/v WST-8 containing medium for 3 h at 37°C under 5 percent CO_2 humidified atmosphere. After 3 h, supernatant was transferred to another 96-

well plate and absorbance values were measured at 450 nm by a microplate reader ELx800 Absorbance Reader (Biotek, USA). As a positive control, 10 percent dimethyl sulfoxide (DMSO) was used.

3.2.5. Uptake Studies

Endocytosis pathways used for the internalization of AuNPs were investigated after 2 h treatment of inhibitors and 22 h treatment of AuNPs using flow cytometry. Briefly, 60,000 cells/well were seeded on each well of 24-well plates in triplicate and incubated 24 h for attachment at 37°C under 5 percent CO₂ humidified atmosphere. After 24 h, cells were exposed to inhibitors with mentioned concentrations for 2 h then inhibitor containing media was removed and fresh media including 25 percent v/v AuNPs were added for treatment groups.

Three different type of the control groups were used for the normalization of the obtained side scatter (SSC) shift from the cells. First of all, the cells without treatment of AuNPs and inhibitor were used to adjust the gate in the flow cytometry as minimum SSC shift originating from the cellular population. Second, the cells treated with only AuNPs were used to see the maximum SSC shift of the cells with AuNPs uptake, and used to adjust obtained SSC shift as 100 percent uptake. Third, the cells treated with only inhibitors were used to eliminate the SSC shift originating from the inhibitors not from the internalized AuNPs. When cells were treated with the inhibitors, and then AuNPs (the treatment group), the SSC shift originating from the inhibitors were subtract from the treatment group, and the resulted SSC shift was adjusted as percent uptake with the use of only AuNPs treated control cells. Side scatter shift (SSC) of the cells was analyzed without further staining as 20,000 events on Guava easy-Cyte 5 (Merck Millipore, USA).

For the uptake of AuNPs by RAW 264.7 cells, 500,000 cells/well were seeded on each well of 24 well plates in triplicate and incubated 24 h for attachment at 37°C under 5 percent CO₂ humidified atmosphere. After 24 h, cells were exposed to only 25 percent v/v AuNPs (Only Au), only LPS (Only LPS), LPS and AuNPs simultaneously (LPS+Au) and first LPS then AuNPs (LPS-Au). After treatments, side scatter shift (SSC) of the cells was analyzed without further staining as 20,000 events on Guava easy-Cyte 5 (Merck

Millipore, USA). As control cells, non-treated cells were used and obtained results were given as SSC shift.

3.2.6. Apoptosis/Necrosis Assay

For the investigation of the induced cell death mechanism by ATP depletion inhibitors (NaN_3 and dG) which are used for receptor mediated endocytosis, Annexin V-FITC apoptosis and necrosis detection kit from Calbiochem (Merck Millipore) was applied according to the manufacturer's instruction. Beas-2b, A549, HeLa and PNT1A cells were seeded on each well of 24-well plates with 60,000 cells/well density in triplicate. Cells were incubated for 24 h for attachment at 37°C under 5 percent CO_2 humidified atmosphere. Then, cells were exposed to 10-100 mM NaN_3 and 10-100 mM dG for 2 h. After 2 h treatment of inhibitors, inhibitor containing media was removed and fresh media was added and incubated for 22 h.

For the investigation the induced cell death mechanism by AuNPs on RAW 264.7 cells, 500,000 cells/well were seeded on each well of 24-well plates in triplicate and incubated 24 h for attachment at 37°C under 5 percent CO_2 humidified atmosphere. After 24 h, cells were exposed to increasing concentrations (25, 50, 60, 70 80 percent v/v) AuNPs.

After 24 h incubation, cells were detached and collected into eppendorf tubes with floating cells in the cell culture medium. The samples were washed with 1X PBS and centrifuged at 1500 rpm for 5 min. Cells were suspended in 1X binding buffer containing 0.5 μl Annexin V-FITC reagent and 1 μl PI reagent per sample and incubated at dark for 15 min according to manufacturer's instruction. Cells were counted as 20,000 events and analyzed on Guava easy-Cyte 5 (Merck Millipore) benchtop flow cytometer. As a positive control, 10 percent DMSO was used.

3.2.7. ATP Assay

Intracellular ATP was quantified using ATP determination kit according to manufacturer's protocol after treatment with NaN_3 or dG. Briefly, Beas-2b, A549, HeLa and PNT1A cells were seeded on each well of 6-well plates with 300,000 cells/well density in triplicate.

Cells were incubated for 24 h for attachment at 37°C under 5 percent CO₂ humidified atmosphere. Then, cells were exposed to 10-100 mM NaN₃ or 10-100 mM dG for 2 h. After 2 h treatment of inhibitors, inhibitor containing media was removed and fresh media was added and incubated for 22 h. After 24 h incubation, cells were harvested and washed with 1X cold PBS. Then, cells were resuspended in ATP assay buffer and centrifuged 5 minutes at 4°C at 13,000 *g*. Obtained supernatants were collected and mixed with 1:1 v/v reaction mix which includes ATP probe, ATP converter and developer mix in ATP assay buffer. Samples were incubated at room temperature for 30 minutes at dark. After incubation, absorbance of the mixture was determined at 570 nm by a microplate reader (ELx800 Absorbance Reader, Biotek). The amount of ATP in the test samples was calculated using ATP standard curve.

3.2.8. Endosome Isolation and Characterization

For the isolation of endosomes, sucrose-Percoll density gradient was used [368]. HeLa cells were seeded to T150 flasks at 5x10⁶ cells/flask density and incubated for 24 h for attachment at 37°C under 5 percent CO₂ humidified atmosphere. Then, cells were exposed to 25 percent (v/v) AuNPs for 15 min, 30 min, 1 h, 4 h, 16 h and 24 h. After incubation with AuNPs, cells were washed with PBS and resuspended in 1 mL homogenization buffer (HB) (0.25 M sucrose, 3 mM Imidazole and 0.5 mM EDTA, pH 7.3) and centrifuged at 750 *g* for 7 minutes. Obtained pellet was resuspended in 200 µL HB and cells were broken with a syringe. The broken cells were checked with light microscope to see the nuclei is still intact. Volume of the obtained homogenate was adjusted to 1 mL and centrifuged 750 *g* for 7 min. For the separation of nuclei from gold filled fragment, obtain pellet was resuspended in 2.5 mL 17 percent Percoll and loaded top of the 2 mL 64 percent sucrose cushion in the Beckman 13.2 mL ultraclean tubes and centrifuged 27,000 *g* for 30 min in a Beckman SW41Ti rotor. After centrifugation, nuclear fraction is remained at the top and gold-filled organelles were found at the bottom.

Isolated endosomes were characterized by DLS from directly pellet obtained from sucrose-Percoll gradient. Moreover, scanning electron microscope (SEM) images were also taken after fixing the endosomes with paraformaldehyde (PFA).

3.2.9. SDS-PAGE and Western Blotting

For the Western-Blotting experiments, membrane of the obtained endosomes was broken with the use of RIPA Buffer and samples were centrifuged at 15,000 g for 30 min. After centrifuge, obtained samples were kept at -80°C for further use in Western-Blotting. Before gel loading, protein amount was determined by BCA assay and each sample was mixed with 4X Laemmli Buffer. 40 µg of total protein was subjected to 6 percent and 15 percent SDS-PAGE and gel run at constant 25 mA with cold running buffer (RB, 25 mM Tris-base, 192 mM glycine, 0.1 percent SDS, pH 8.5).

For the determination of proteins adsorbed on AuNPs, AuNPs were centrifuged at 14,000 rpm for 10 min and washed twice. And then protein amount was determined by BCA assay and equal amounts of proteins were loaded to the 12 percent SDS-PAGE gel and run at constant 25 mA. After running the gel two different staining protocols Coomassie blue and silver staining methods were tried.

For Coomassie blue staining, gels were washed and stained overnight at room temperature with Coomassie Blue and then, destained with destaining buffer (50 percent v/v methanol in water with 10 percent v/v acetic acid) for 4-5 h. Images were taken by ImageLab imaging system (Biorad, USA). For silver staining, gels were washed and fixed overnight at room temperature with fixing solution (50 percent v/v methanol, 12 percent v/v acetic acid and 0.0185 percent v/v formaldehyde). After fixing, gels were washed with wash buffer (50 percent v/v methanol in dH₂O) for 20 min three times and then, washed with water five times. After washing steps, 1.3 mM Na₂S₂O₃ solution was poured onto gels and incubated rotating for 1 minute. Then, gels were washed again five times with water and stained by 11.8 mM AgNO₃ solution including 0.028 percent v/v formaldehyde for 20 min at +4°C. After staining, gels were placed in a new box and washed five times with water then developing solution (6 percent w/v Na₂CO₃, 0.05 mM Na₂S₂O₃, and 0.037 percent v/v formaldehyde) was poured onto gels and swirl until desired staining intensity was achieved. Then, gels were washed and stop solution (50 percent v/v methanol and 12 percent v/v acetic acid) was added onto gels and images of the gels were taken by ImageLab imaging system [369, 370].

For western blotting, two different conditions were used for small and big proteins. For the transfer of small proteins, (GAPDH, 37 kDa, Rab7, 23kDa and Rab11, 25 kDa), percentages of the ingredients in the transfer buffer (TB, 25 mM Tris-Base, 192 mM glycine, pH 8.3) was adjusted as 20 percent methanol, 10 percent 10X TB 70 percent dH₂O. For big proteins (EEA1, 170 kDa, GM-130, 130 kDa, LAMP1, 120 kDa, calnexin, 90 kDa) TB percentages were optimized as 25 percent methanol, 10 percent 10X TB, 65 percent dH₂O. Small proteins were transferred to PVDF membranes at constant 125 mA for 90 min and big proteins were transferred at 70V for 4 h. After transfer, PVDF membranes were placed into blocking solutions at room temperature for 1 h. After blocking, membranes were incubated with mouse anti-Rab7 primary antibody, rabbit anti-GAPDH primary antibody, rabbit anti-Rab11 primary antibody, rabbit anti-LAMP1 primary antibody, rabbit anti-EEA1 primary antibody, rabbit anti-calnexin primary antibody and rabbit anti-GM-130 primary antibody overnight at 4°C. After overnight incubation, for better binding of the antibody to bigger proteins, incubation of GM-130, EEA1, LAMP1 and calnexin antibodies were continued at room temperature for 1 h. After primary antibody incubation, membranes were washed with TBS-T buffer (1 M Tris-HCl, 1.5 M NaCl with 0.5 percent Tween-20, pH 7.4) and blocked for 20 min and exposed to secondary antibodies for 2 h at room temperature. After secondary antibody incubations, membranes were washed again with TBS-T and PBS and blotting images were obtained using ImageLab imaging system (Biorad, USA).

3.2.10. Macrophage Activation and Nitrite Detection

For the activation, RAW 264.7 cells were seeded on 96-well-plate in triplicate at 100,000 cell/well density. Cells were incubated for 24 h for attachment at 37°C under 5 percent CO₂ humidified atmosphere. Then, cells were stimulated with 50-1000 µg/mL of lipopolysaccharide (LPS) from *E. coli* (0111:B4, Sigma, USA) for 24 h with or without AuNPs.

AuNPs were used with LPS (LPS+Au) and also after LPS treatment (LPS-Au) to investigate macrophage activation mechanism and also AuNPs response after activation, respectively. For the investigation of macrophage activation, 25 percent v/v AuNPs were added to the medium including different concentration of LPS (50, 100, 250, 500, 1000

µg/mL). For the investigation of AuNPs response after activation, cells were firstly activated with 1000 µg/mL LPS for 24 h. Activation was checked with Griess reagent for nitrite detection and then old media is removed, fresh media including 25 percent v/v AuNPs were added onto cells and incubated for another 24 h.

After treatments with LPS and/or AuNPs, nitrite was detected from the supernatant using Griess reagent [371]. Supernatant was mixed with Griess reagent (1 percent sulfanilamide and 0.1 percent N-(1-naphthyl) ethylenediamine dihydrochloride in 5 percent phosphoric acid) and incubated at room temperature for 10 min. After incubation, absorbance of the mixture was determined at 540 nm using a microplate reader (ELx800 Absorbance Reader). 100 µM N(G)-Nitro-L-arginine methyl ester (L-NAME) was used as positive control.

3.2.11. Cellular Reactive Oxygen Species (ROS) Assay

For the determination of ROS in the macrophages, RAW 264.7 cells were seeded in 24-well-plate in triplicate at 500,000 cell/well density. Cells were incubated for 24 h for their attachments at 37°C under 5 percent CO₂ humidified atmosphere. After attachment, cells were incubated with different concentrations of LPS (50,100, 250, 500, 1000 µg/mL) with or without AuNPs for 24 h. Then, cells were harvested, collected in eppendorf tubes and washed with PBS and stained with 25 µM DCFDA and incubated for 30 min at 37°C. After staining, cells were counted as 20,000 events and analyzed on Guava easy-Cyte 5-benchtop flow cytometer. As a positive control, 1 percent DMSO was used.

3.2.12. SERS Measurements

Beas-2b, A549, HeLa and PNT1A cells were seeded on approximately 1 cm² calcium fluoride (CaF₂) slides in 24-well plates with 15,000 cells/well density. Cells were incubated and treated with inhibitors and AuNPs as mentioned above. After the treatment, AuNPs containing media was removed, cells were washed with PBS, and the cells attached to CaF₂ slides were placed on polydimethylsiloxane (PDMS) coated Petri dish to prevent polystyrene spectral background. To prevent cells drying, 20 µL cell culture medium was

added on CaF₂ slides while measurement. Illustration of the experimental design for SERS measurements are given in Figure 3.1.

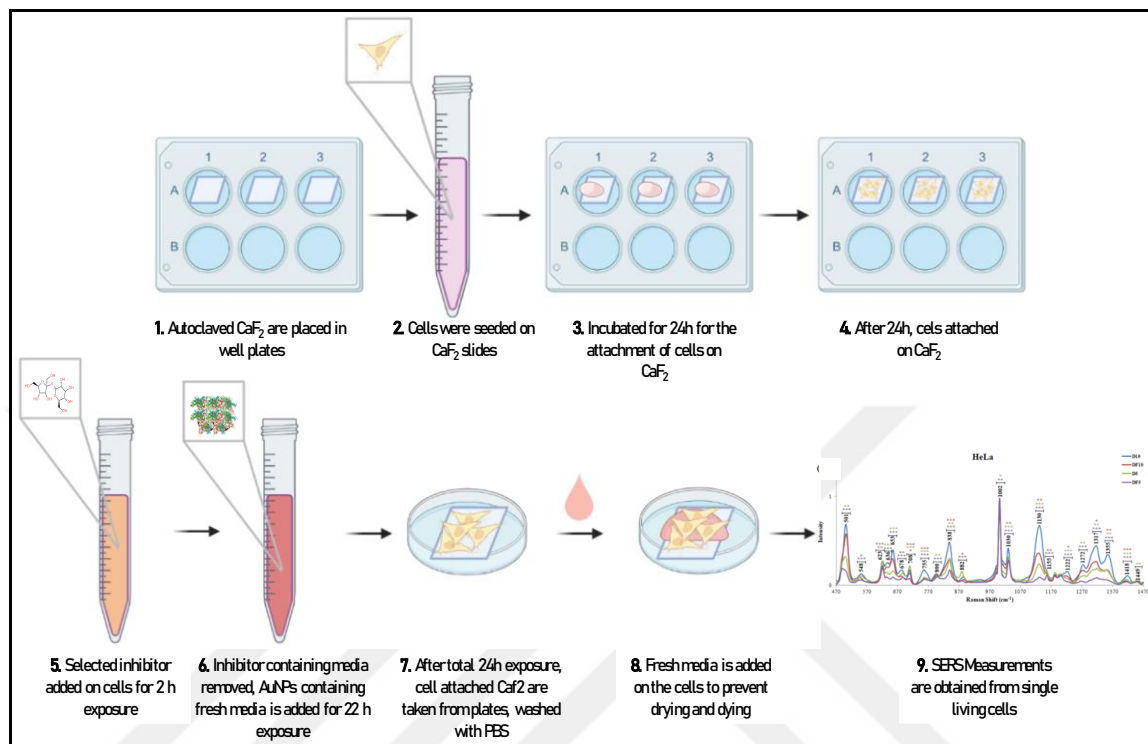


Figure 3. 1.Experimental setup for SERS measurements

RAW 264.7 cells were seeded on 96-well-plate with 100,000 cells/well density to allow LPS activation [371]. Cells were treated with LPS and/or AuNPs as mentioned above. After the treatment, the cells were scraped from the wells and placed on CaF₂ slides with 20 μ L cell culture medium.

For measurements, Renishaw inVia Reflex Raman spectrometer equipped with a high speed encoded stage (Streamline) and a Leica DM2700 Dark Field upright microscope with an 830 nm laser source and 1200 line/mm grating was used. For the mapping of cells, approximately 10 μ m x 10 μ m area was mapped with a 2 μ m step size due to a 2.5 μ m of laser spot size for Leica 20x objective with 0.40 NA. Spectra from the cells were collected with 150 mW laser power for 2 s exposure time in the spectral range of 470-1470 cm⁻¹. From each cell, 42-64 spectra collected and averaged. For each treatment group, minimum 10 cells were used for spectral collection for one experiment, and each experiment was run in triplicate. Obtained spectra from 30 cells for each treatment were averaged and

processed for background correction, smoothing, removal of cosmic spikes and normalization using Wire 4.2 software.

To investigate the interfere of SERS spectra from inhibitors, highest concentration of each inhibitor (100 mM NaN₃, 100 mM dG, 100 μ M Aml, 10 μ M CytoD, 500 mM Scr, 100 mM CPM, 2 μ M Fil, 0.4 μ M Nys and 80 nM Dyn) was mixed with 25 percent v/v AuNPs and dropped on CaF₂ slides and dried. After drying, SERS spectra of inhibitors were obtained by 10 s exposure time with 150 mW laser power. 10 spectra were collected for each inhibitor and obtained spectra were analyzed as same with spectra obtained from cells.

3.2.13. Statistical Analysis

For the emphasis of the variation of SERS spectra, principal component analysis (PCA) was applied to the obtained average SERS spectra from 30 cells for each treatment group. After PCA analysis, linear discriminant analysis (LDA) was applied to the obtained PC scores to observe the discrimination of the obtained spectra with treatments of inhibitors. Two-tailed student t-test was applied to cytotoxicity, uptake, Apoptosis/Necrosis assay, ROS assay, ATP assay results. Two-tailed student t-test was also applied to intensity of desired Raman shift which is given as Table 4.3.-4.6. and obtained p-values from the comparison of the control group and the highest dose were shown on the SERS spectra of belonging treatment.

4. RESULTS AND DISCUSSION

4.1. AuNP CHARACTERIZATION

In this study, citrate reduced 50 nm average size AuNPs was used thanks to their not only highest uptake by cells but also high SERS enhancement properties [209,308,311]. Synthesized AuNPs were characterized with UV/Vis spectroscopy and Dynamic Light Scattering (DLS) as shown in Figure 4.1. Maximum absorbance was obtained at 530 nm (Figure 4.1.a) which is a proof of approximately 50 nm size of AuNPs [372]. Moreover, hydrodynamic size of AuNPs were found as 59 nm (Figure 4.1.b) which is larger than estimated size due to the obtained hydrodynamic diameter measured with citrate ions associated on the AuNPs surface.

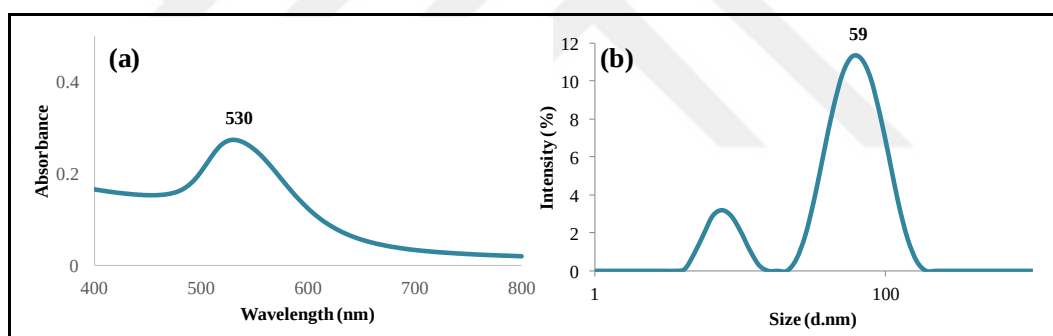


Figure 4.1. AuNP Characterization. (a) UV/Vis spectrum and (b) DLS spectrum

4.2. PROTEIN CORONA STUDIES

When nanomaterial interaction with living systems is investigated, nanomaterials cannot be thought as bare nanoparticles. When they are added to the cell culture media or blood, immediately a layer is formed which is called as protein corona (PrC). PrC determines the fate of the nanomaterials including cytotoxicity, uptake and clearance. Thus, investigation of the PrC has an important role to understand NM-living system interactions. In this study, protein corona on the AuNPs was investigated and their effect on the obtained SERS spectra from AuNPs and also cells were examined.

4.2.1. Protein Corona Characterization

Formation of protein corona (PrC) was characterized by the UV/Vis spectroscopy and DLS as seen in Figure 4.2.-4.7. and in Table 4.2.-4.5. Protein corona is determined as soft corona (SC) and hard corona (HC). SC measurements were obtained *in situ* from cell culture medium without any further sample preparation or AuNPs separation. On the other hand, HC measurements obtained after centrifugation. With centrifugation, SC is eliminated and remaining tightly bonded proteins are referred as HC.

Figure 4.2. shows UV/Vis spectra of bare AuNPs and AuNPs incubated with different cell culture media and FBS in increasing time points. It was found that even AuNPs were added to the cell culture ($t=0$), protein corona formation starts immediately and red shift in the surface plasmon resonance (SPR) of AuNPs could be observed and there was not any SPR shift change with increasing time for SC. This could be explained by the rapid formation of protein corona which starts within 30 s after NPs were added to the biological environment [285]. Although with increasing time, protein profile changes based on the protein composition of the medium [373], these changes could not tracked by UV/Vis spectra. Obtained maximum absorbance values were higher than the bare AuNPs which indicates the dense dielectric layer formation formed by protein adsorption; however, when time points were compared to each other, the maximum absorbance values were similar at increasing time points showing the direct formation of AuNPs starting from the adding AuNPs to the media.

Furthermore, even protein profile changes could not be observed by UV/Vis, these changes could be tracked by SDS-PAGE images as shown in Figure 4.9. On the other hand; even there was not any significant shift in the SPR peak over time, the achievement of the similar SPR shift gives information about the stability of the NPs in the medium over time [220]. Lack of loss or big shifts in the SPR peak shows the formation of a protein layer protected by time and provides stability which prevents the nanoparticles aggregation in all type of cell culture conditions

Under different experimental conditions; slight differences were observed at the SPR of SC coated AuNPs for different media and FBS conditions. Highest SPR shift was achieved by DMEM type cell culture media and lower shift was observed with DF12 cell culture media

including 5 percent FBS. Concentration of the FBS did not provide any significant change while using different type of cell culture media resulted as differences in the obtained absorbance peaks. With the use of DMEM, sharper absorbance peak was obtained when compared to DMEM:F12. This situation can be explained with the differences in the composition of two different media as shown in Table 4.1. In the literature, Maiorano et al. showed that composition of the cell culture media affects directly the hydrodynamic size of the PrC-NPs complex [374]. They used NaCl and glucose as main components in the cell culture media and found that when ionic strength and glucose concentration decrease, hydrodynamic size decreases. They have also showed that with the use of DMEM type media, obtained absorbance band intensity increases over time and larger spectral changes were observed when compared with RPMI due to the more abundant protein clustering with higher amount of protein in the PrC structure. Casals et al. also reported that increased ionic strength could cause rapid aggregation and precipitation [220]. When DMEM and DF12 were compared based on the composition and concentrations as shown in Table 4.1., all ingredients differ in both composition and concentration-based manner. With higher salt concentration in the DF12 type of cell culture media, due to the increased aggregation ability of the NPs in the surrounding environment it could provide more broadened SPR peak [220]. It should be also noted that not only glucose or salt concentration difference but also differences in other components in the cell culture media could affect the PrC formation and provide spectral changes.

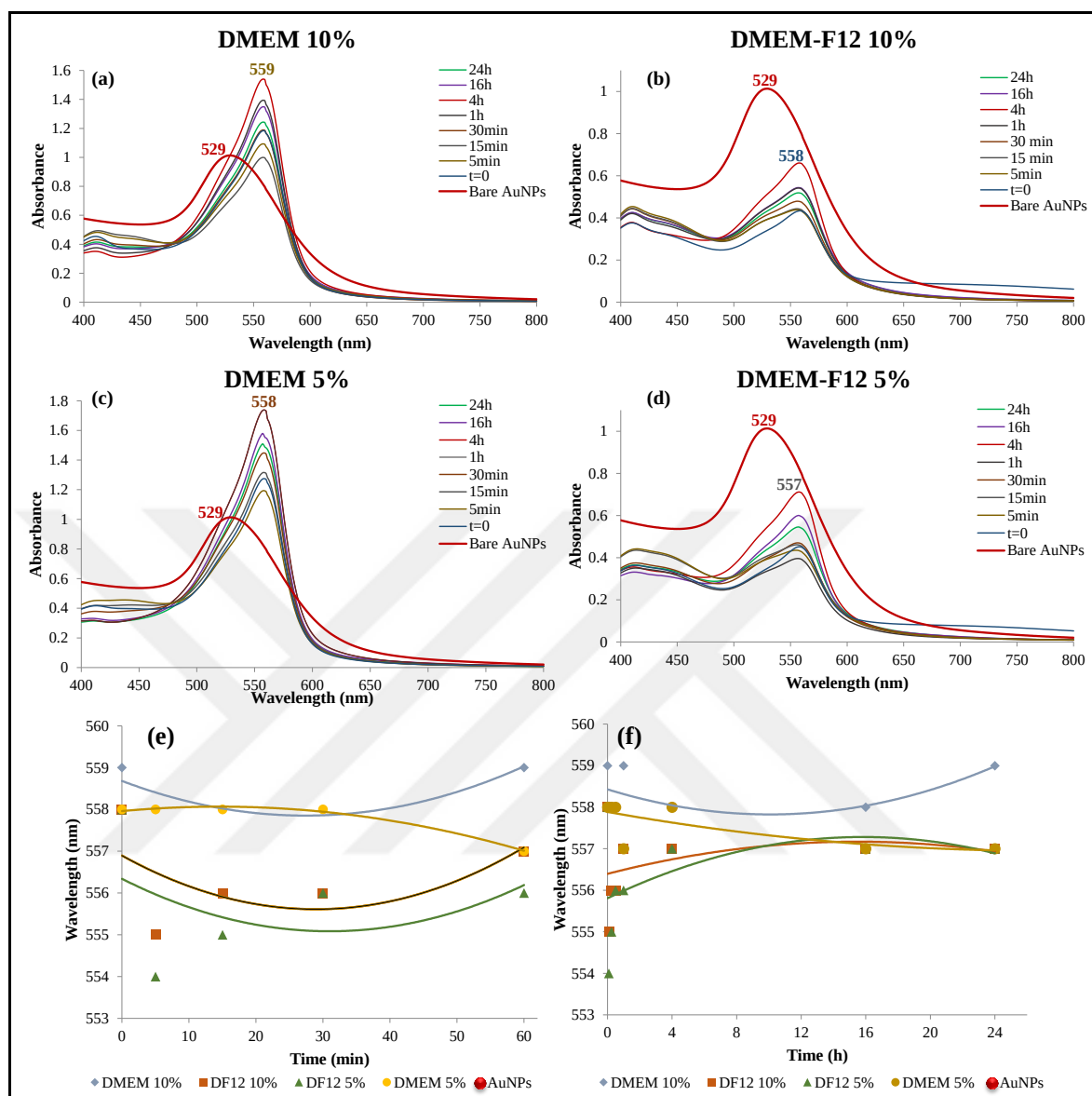


Figure 4.2. UV/Vis Spectra of AuNPs without any centrifugation (Soft Corona, SC) in (a) DMEM with 10 percent FBS, (b) DMEM-F12 with 10 percent FBS, (c) DMEM with 5 percent FBS and (d) DMEM-F12 with 5 percent FBS. (e), (f) Time dependent SPR band shifts (e) 0-1 h and (f) 0-24 h

Table 4.1. Compositions of DMEM and DMEM:F12 cell culture media

	DMEM (mg/L)	DMEM:F12 1:1 (mg/L)
Amino Acids		
Glycine	30.0	18.75
L-Arginine hydrochloride	84.0	147.5
L-Cystine 2HCl	63.0	31.29
L-Glutamine	584.0	-
L-Histidine hydrochloride-H ₂ O	42.0	31.48
L-Isoleucine	105.0	54.47
L-Leucine	105.0	59.05
L-Lysine hydrochloride	146.0	91.25
L-Methionine	30.0	17.24
L-Phenylalanine	66.0	35.48
L-Serine	42.0	26.25
L-Threonine	95.0	53.45
L-Tryptophan	16.0	9.02
L-Tyrosine disodium salt dihydrate	104.0	55.79
L-Valine	94.0	52.85
L-Alanine	-	4.5
L-Asparagine.H ₂ O	-	7.5
L-Aspartic acid	-	6.65
L-Cysteine.HCl.H ₂ O	-	17.56
L-Glutamic acid	-	7.35
L-Proline	-	17.25
Vitamins		
Choline chloride	4.0	8.98
D-Calcium pantothenate	4.0	2.24
Folic Acid	4.0	2.65
Niacinamide	4.0	2.02
Pyridoxine hydrochloride	4.0	2.031
Riboflavin	0.4	0.219
Thiamine hydrochloride	4.0	2.17
i-Inositol	7.2	12.6
D-Biotin	-	0.0035
Vitamin B12	-	0.68

Inorganic Salts		
Calcium Chloride (CaCl_2)	200.0	154.5
Ferric Nitrate ($\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$)	0.1	0.05
Magnesium Sulfate (MgSO_4)	97.67	48.84
Potassium Chloride (KCl)	400.0	311.8
Sodium Bicarbonate (NaHCO_3)	3700.0	1200.0
Sodium Chloride (NaCl)	6400.0	6996.0
Sodium Phosphate monobasic ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$)	125.0	54.3
Copper sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)	-	0.0013
Iron(II) sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)	-	0.417
Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$)	-	61.2
Sodium Phosphate (Na_2HPO_4)	-	71.02
Zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$)	-	0.432
Other Components		
D-Glucose (Dextrose)	4500.0	3150.0
Phenol Red	15.0	8.63
Sodium Pyruvate	110.0	55
HEPES	-	35.745
Hypoxanthine	-	2.44
Linoleic Acid	-	0.042
Putrescine.2HCl	-	0.081
DL-Thioctic Acid	-	0.105
Thymidine	-	0.365

Figure 4.3. shows hydrodynamic size of the AuNPs in different cell culture media, and as in the UV/Vis spectra, SC formation on the AuNPs surface immediately after adding into the cell culture can be observed under all conditions. Time dependent significant changes in the hydrodynamic sizes of AuNPs were not observed. On the other hand, when concentrations of the FBS in the cell culture media were compared, the observed increase in hydrodynamic size of AuNPs indicates increased thickness of the SC with increasing concentration of FBS. With the use of different cell culture media, size shift also changed, and a thicker SC formation was observed with DMEM as cell culture medium. Consequently, similar to the reports in the literature [374-377], it was concluded that increasing FBS concentration increases SC layer thickness on the AuNPs surface and cell culture media affects the formation of SC.

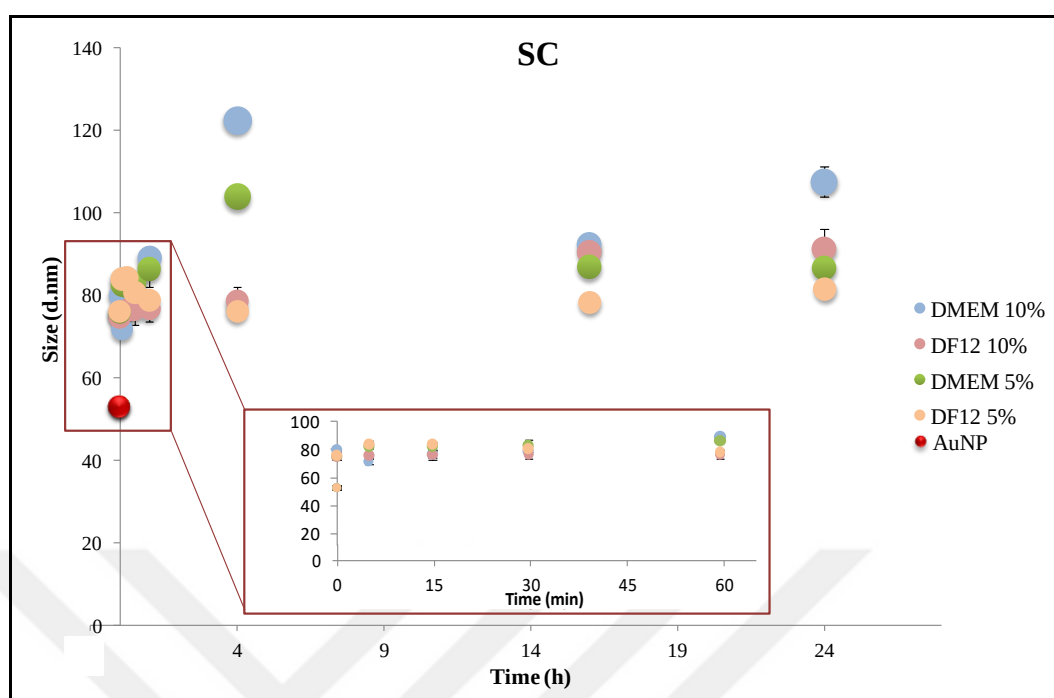


Figure 4.3. Hydrodynamic sizes of AuNPs without any centrifugation (SC) in DMEM with 10 percent FBS, DMEM-F12 (DF12) with 10 percent FBS, DMEM with 5 percent FBS and DMEM-F12 (DF12) with 5 percent FBS

When the formation of SC was examined by tracking the zeta potential shifts as seen in Figure 4.4., the similar results as found from the hydrodynamic size and UV/Vis spectroscopy study was found, where AuNPs were coated immediately with proteins after adding to the cell culture resulted in a sudden zeta potential increase up to -6.49 mV for DMEM with 10 percent FBS, -7.75 mV for DMEM-F12 with 10 percent FBS, -8.55 mV for DMEM with 5 percent FBS and -10.16 mV for DMEM-F12 with 5 percent FBS. When different cell culture conditions were compared to the zeta potential values coating slightly changed by SC protein coating on the AuNPs surface.

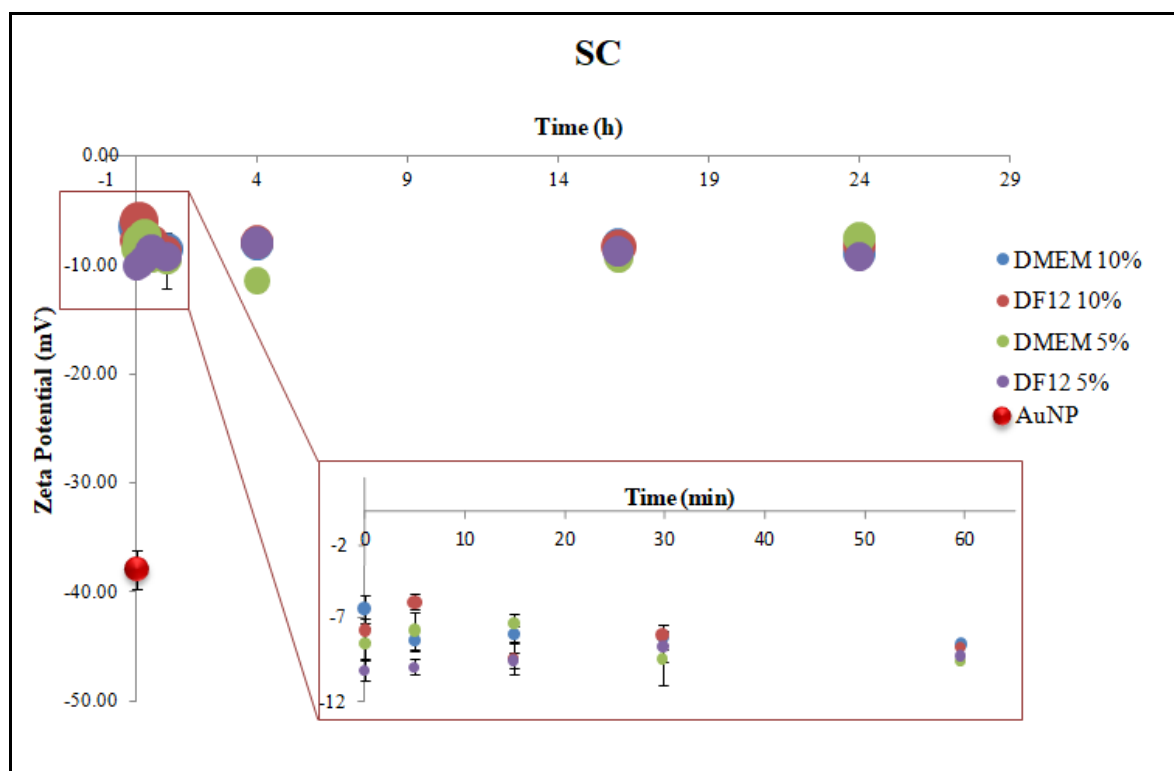


Figure 4.4. Zeta Potential of AuNPs without any centrifugation (SC) in DMEM with 10 percent FBS, DMEM-F12 (DF12) with 10 percent FBS, DMEM with 5 percent FBS and DMEM-F12 (DF12) with 5 percent FBS

The characterization of HC was obtained by the elimination of SC using centrifugation. HC was characterized by UV/Vis spectra and DLS measurements as seen in Figure 4.5.-4.7. In Figure 4.5., obtained UV/Vis spectra of AuNPs in different cell culture media and FBS concentrations are shown and it can be seen that obtained red shift decreases when compared to the SC caused red shift which means thickness of the PrC is decreased by the desorption of SC proteins. When cell culture media dependent change of SPR is considered, DF12 type of the cell culture caused smaller red shift and concentration of the FBS cause slight changes in the SPR of AuNPs as seen in Figure 4.5.e and 4.5.f.

Consequently, it can be said that differences in the cell culture media and FBS concentration alters the HC conformation which represented in the changed SPR in UV/Vis spectra of AuNPs.

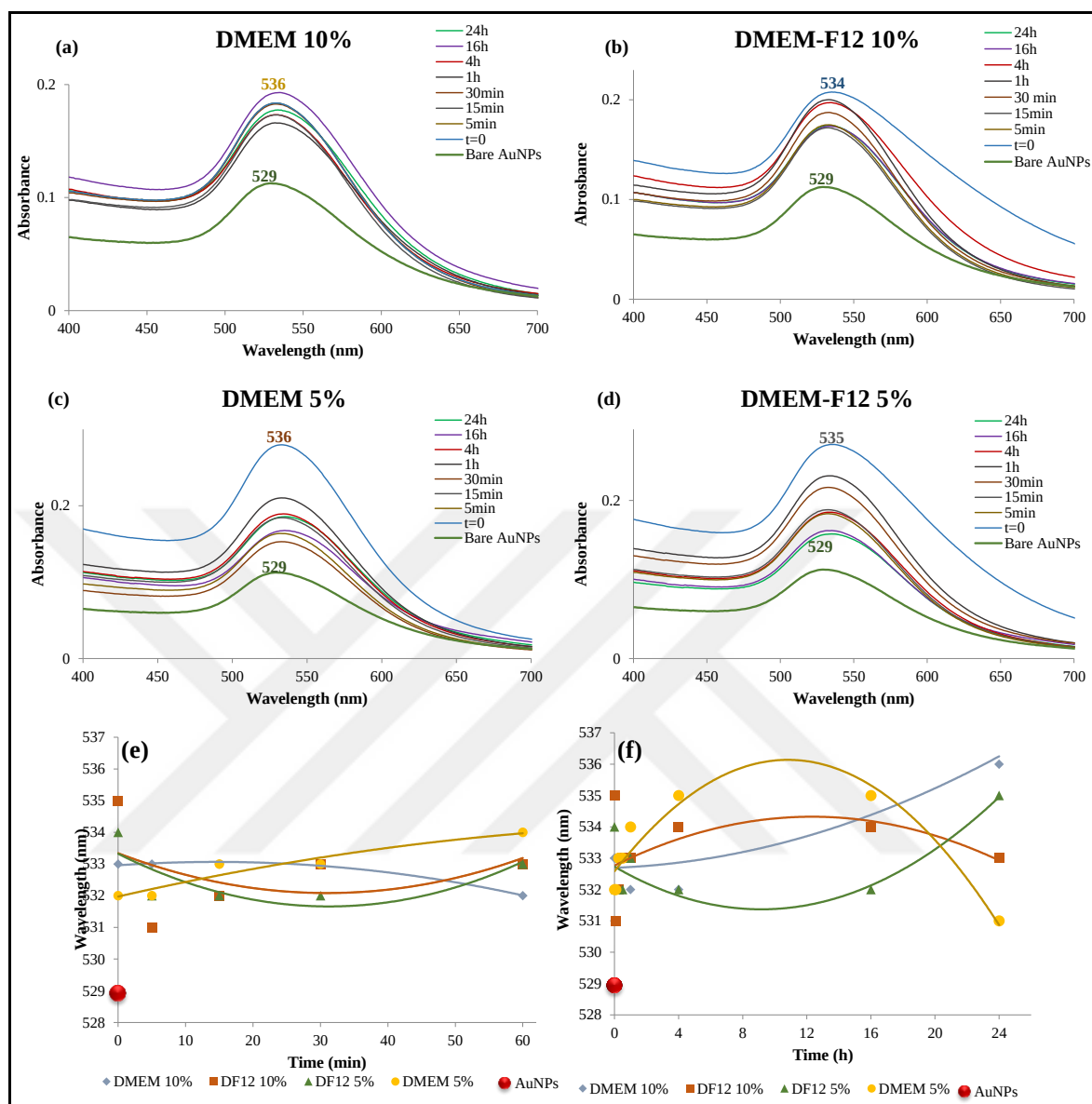


Figure 4.5. UV Spectrum of AuNPs after centrifugation (Hard Corona, HC) in (a) DMEM with 10 percent FBS, (b) DMEM-F12 with 10 percent FBS, (c) DMEM with 5 percent FBS and (d) DMEM-F12 with 5 percent FBS. (e), (f) SPR shift of AuNPs coated with HC in time points between (e) 0-1 h and (f) 0-24 h

Hydrodynamic size changes of AuNPs coated with HC in different cell culture media, FBS concentration and time were measured and showed in Figure 4.6. and Table 4.2.-4.5. As seen, HC formation is time dependent and affected by the cell culture media type and FBS concentration. It was found that when SC was eliminated by centrifugation, despite lower absorption peak was achieved with UV absorption spectrum due to the decreased amount

of protein with the dissociation of low affinity proteins from the AuNPs surface, smaller hydrodynamic sizes was not achieved. This could be caused by the changed surrounding environment by centrifugation because SC measurements were obtained ex situ inside the cell culture media while HC measurements were obtained in the distilled water after centrifugation step.

When cell culture media types were compared, higher hydrodynamic sizes were measured like in the SC measurements for DMEM. Similar with the SC, differences in the hydrodynamic size which is the result of altered PrC formation could be related to the composition of the cell culture media. With the use of different cell culture media, AuNPs expose to the different molecules and different concentrations of the same components and this could alter the protein adsorption and also desorption. Moreover, when FBS concentration increases, similar with SC measurements, hydrodynamic size increases which means FBS concentration affects positively the thickness of the HC.

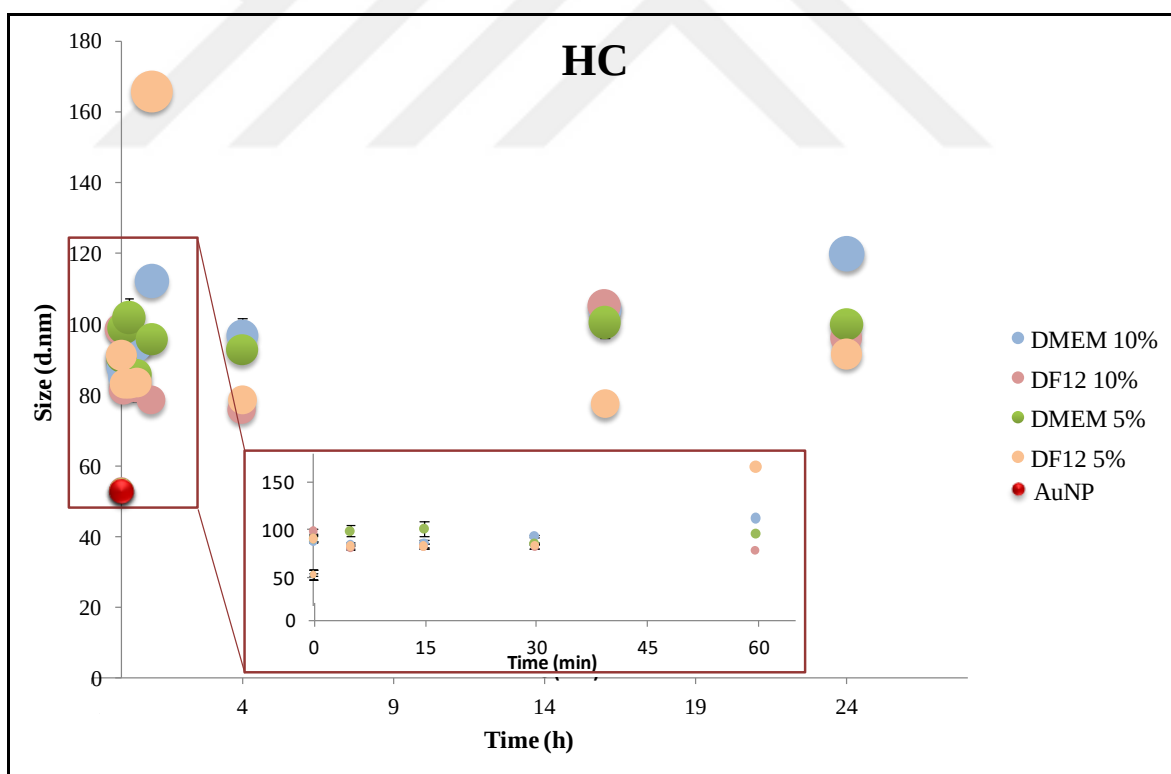


Figure 4.6. Hydrodynamic sizes of AuNPs after centrifugation (HC) in DMEM with 10 percent FBS, DMEM-F12 (DF12) with 10 percent FBS, DMEM with 5 percent FBS and DMEM-F12 (DF12) with 5 percent FBS

Figure 4.7. shows zeta potential measurements of AuNPs coated with HC in different cell culture media and FBS concentrations. Zeta potential changes were also found as time dependent, similar to hydrodynamic size measurements and when compared to SC measurements, obtained surface charges totally differs when SC is eliminated from the surface of the AuNPs. The zeta potential value comes close to the value of bare AuNPs with the adsorption of only hard corona proteins which has long residence time and high affinity to the AuNPs surface. With the use of different cell culture media and FBS concentration, slight differences were observed at the zeta potential measurements.

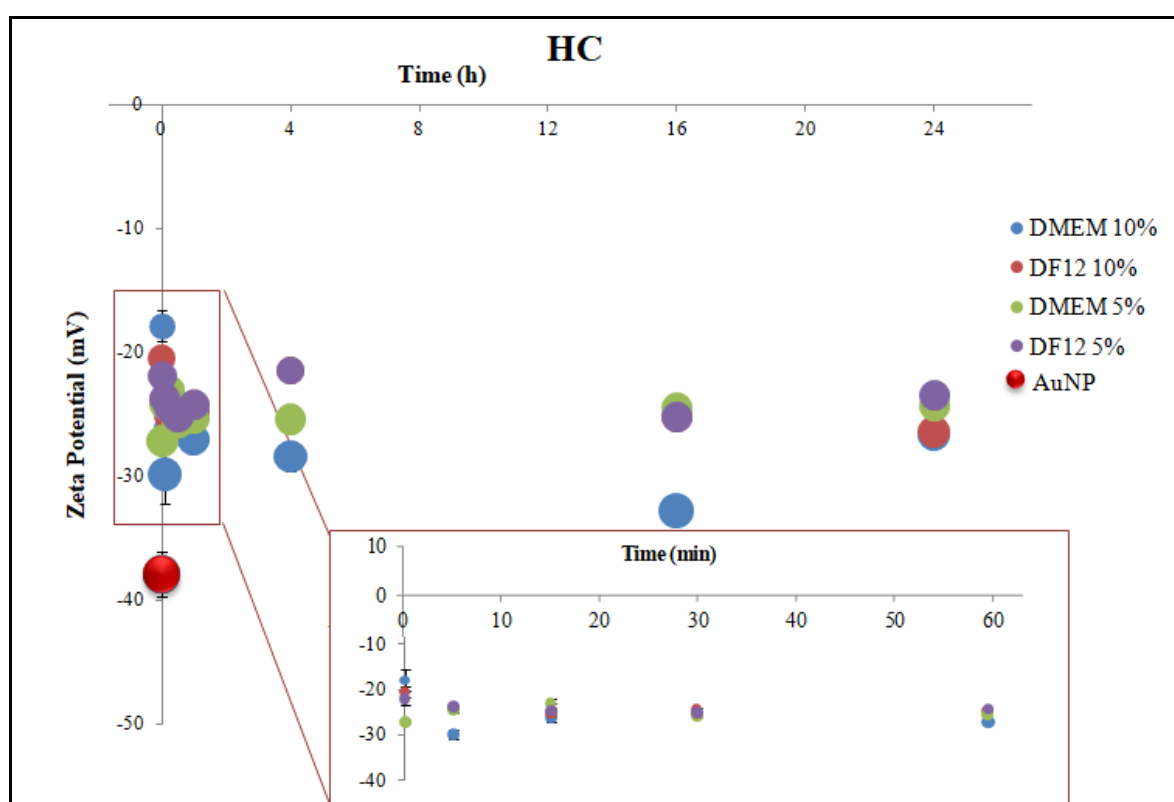


Figure 4.7. Zeta Potential of AuNPs after centrifugation (HC) in DMEM with 10 percent FBS, DMEM-F12 (DF12) with 10 percent FBS, DMEM with 5 percent FBS and DMEM-F12 (DF12) with 5 percent FBS

Table 4.2. Average hydrodynamic sizes and zeta potentials of bare AuNPs and in DMEM with 10 percent FBS

DMEM 10%		SC		HC	
		Size (d.nm)	Zeta P. (mV)	Size (d.nm)	Zeta P. (mV)
Bare AuNPs		53.21 ± 1.02	-37.9 ± 1.77	53.21 ± 1.02	-37.9 ± 1.77
Time	0	80.03 ± 1.15	-6.49 ± 0.57	87.8 ± 1.18	-17.86 ± 1.26
	5 min	72.61 ± 1.91	-8.36 ± 0.83	85.04 ± 0.93	-29.9 ± 2.34
	15 min	76.92 ± 2.18	-8.0 ± 0.63	85.95 ± 1.25	-26.2 ± 0.92
	30 min	78.72 ± 12.80	-8.13 ± 0.50	93.49 ± 4.2	-25.3 ± 1.00
	1 h	89.03 ± 2.05	-8.58 ± 0.73	112.1 ± 1.87	-27.1 ± 0.20
	4 h	122.4 ± 0.60	-8.17 ± 0.36	96.62 ± 5.05	-28.4 ± 1.12
	16 h	92.25 ± 1.51	-8.13 ± 1.01	104.3 ± 4.15	-32.8 ± 1.18
	24 h	107.6 ± 3.62	-9.00 ± 1.08	119.7 ± 4.1	-26.7 ± 0.58

Table 4.3. Average hydrodynamic sizes and zeta potentials of bare AuNPs and in DMEM-F12 (DF12) with 10 percent FBS

DF12 10%		SC		HC	
		Size (d.nm)	Zeta P. (mV)	Size (d.nm)	Zeta P. (mV)
Bare AuNPs		53.21 ± 1.02	-37.9 ± 1.77	53.21 ± 1.02	-37.9 ± 1.77
Time	0	74.67 ± 1.40	-7.75 ± 0.50	98.4 ± 4.89	-20.46 ± 0.98
	5 min	75.95 ± 1.51	-6.06 ± 0.66	81.30 ± 2.16	-23.9 ± 1.27
	15 min	76.39 ± 1.25	-9.41 ± 0.47	82.63 ± 1.75	-25.1 ± 0.153
	30 min	76.61 ± 3.67	-8.01 ± 1.01	82.07 ± 2.47	-24.5 ± 0.55
	1 h	76.71 ± 3.15	-8.78 ± 0.61	78.40 ± 2.14	-24.9 ± 0.30
	4 h	78.56 ± 3.37	-7.95 ± 0.53	75.82 ± 1.67	-21.5 ± 0.88
	16 h	90.52 ± 1.56	-8.32 ± 1.29	104.9 ± 4.10	-25.2 ± 0.55
	24 h	91.34 ± 4.72	-8.30 ± 0.04	95.83 ± 5.22	-26.4 ± 0.28

Table 4.4. Average hydrodynamic sizes and zeta potentials of bare AuNPs and in DMEM with 5 percent FBS

DMEM 5%		SC		HC	
		Size (d.nm)	Zeta P. (mV)	Size (d.nm)	Zeta P. (mV)
Bare AuNPs		53.21 ± 1.02	-37.9 ± 1.77	53.21 ± 1.02	-37.9 ± 1.77
Time	0	75.76 ± 1.62	-8.55 ± 0.52	90.77 ± 1.70	-27.13 ± 0.5
	5 min	82.51 ± 2.62	-7.75 ± 0.94	98.89 ± 1.85	-24.26 ± 0.45
	15 min	82.15 ± 2.00	-7.34 ± 1.11	101.73 ± 1.91	-23.03 ± 0.43
	30 min	82.95 ± 2.70	-9.45 ± 0.58	85.74 ± 1.61	-25.66 ± 0.48
	1 h	86.48 ± 4.33	-9.57 ± 2.55	95.6 ± 1.79	-25.4 ± 0.47
	4 h	104.0 ± 1.58	-11.43 ± 1.00	92.82 ± 1.74	-25.43 ± 0.47
	16 h	86.89 ± 1.60	-9.37 ± 0.78	100.4 ± 1.88	-24.53 ± 0.46
	24 h	86.63 ± 0.73	-7.58 ± 0.95	99.92 ± 1.87	-24.3 ± 0.45

Table 4.5. Average hydrodynamic sizes and zeta potentials of bare AuNPs and in DMEM-F12 (DF12) with 5 percent FBS

DF12 5%		SC		HC	
		Size (d.nm)	Zeta P. (mV)	Size (d.nm)	Zeta P. (mV)
Bare AuNPs		53.21 ± 1.02	-37.9 ± 1.77	53.21 ± 1.02	-37.9 ± 1.77
Time	0	76.06 ± 1.50	-10.16 ± 0.25	91.15 ± 5.58	-21.93 ± 0.58
	5 min	83.91 ± 1.59	-9.94 ± 3.81	83.23 ± 1.56	-23.76 ± 0.44
	15 min	84.21 ± 2.14	-9.53 ± 3.97	82.97 ± 1.55	-24.53 ± 0.46
	30 min	80.61 ± 1.00	-8.69 ± 4.35	83.59 ± 1.57	-25.16 ± 0.47
	1 h	78.64 ± 1.47	-9.26 ± 4.09	165.73 ± 3.11	-24.23 ± 0.45
	4 h	76.10 ± 1.15	-7.96 ± 4.75	78.67 ± 1.47	-21.5 ± 0.40
	16 h	78.20 ± 1.46	-8.82 ± 4.29	77.42 ± 1.45	-25.23 ± 0.47
	24 h	81.4 ± 1.52	-9.26 ± 4.09	91.68 ± 1.72	-23.46 ± 0.44

4.2.2. Protein Profiling

The adsorption of proteins on the AuNPs surface was investigated by SDS-PAGE. To obtain gel images, different staining protocols: coomassie blue staining and silver staining, different sample volumes: 1 and 5 mL and different sample buffer types: reducing buffer with β -mercaptoethanol (β ME) and non-reducing buffer without β ME were compared as shown in Figure 4.8. As seen, silver staining provided better gel images with clear bands. For sample volume, 5 mL provided too much intense bands thus, 1mL was selected as sample volume and it was also found that reducing sample buffer provided better band vision with the break of all disulfide bonds and denaturation of the proteins. Thus, remaining images were obtained by 1mL sample volume using reducing sample buffer stained with silver staining.

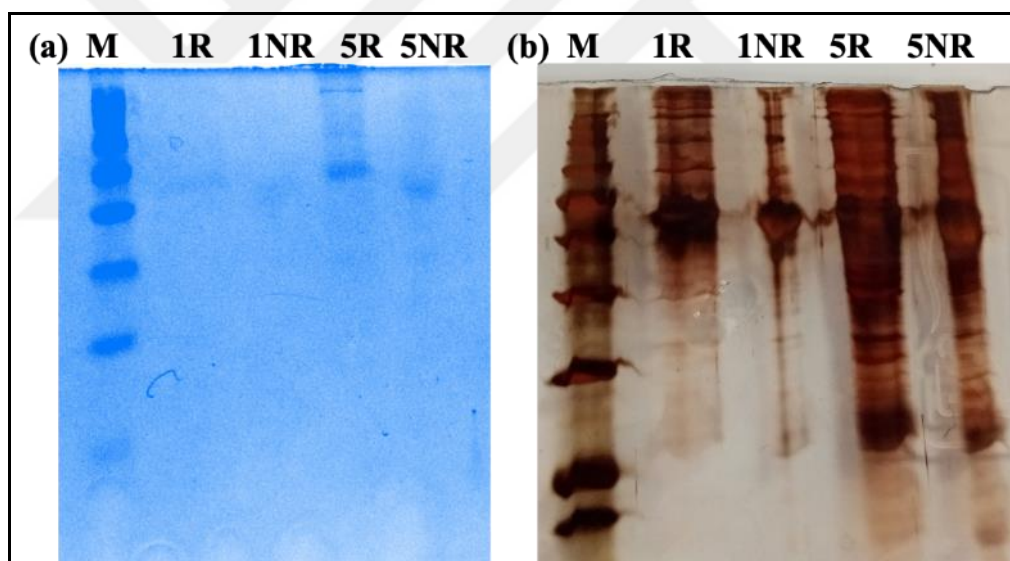


Figure 4.8. Gel images of AuNPs-protein corona samples dyed with (a) Coomassie blue, (b) silver. M: Marker, 1R: 1mL sample volume with reducing sample buffer, 1NR: 1mL sample volume with non-reducing sample buffer, 5R: 5mL sample volume with reducing sample buffer, 5NR: 5mL sample volume with non-reducing sample buffer

After selecting the staining protocol, time dependent development of protein corona in different cell culture media with different FBS concentration was examined by SDS-PAGE and obtained images could be seen in Figure 4. 9. It was found that various proteins were adsorbed on the AuNPs surface. It is known that structure of the PrC is dependent on the

properties of the NMs such as type, size, shape and surface chemistry [378] and various proteins, which has important biological functions such as homeostasis, transport and trafficking, complement activation, blood coagulation, tissue development, have been identified for PrC on the AuNPs surface such as serum albumin (66 kDa), apolipoprotein A1 (28 kDa) and apolipoprotein E (34 kDa), complement C3 (185 kDa) [374,379,380]. Moreover, not only NMs properties but also environmental conditions could alter the PrC conformation such as type of cell culture media, type of protein source or concentration of protein source [374,376]. In Figure 4.9., it could be seen that protein profile changed by time due to the dissociation association of different proteins on AuNPs surface overtime with the use of different cell culture media and FBS concentration. This is an expected result because it is known when nanoparticles interact with the cell culture media including protein source, type and abundance of the proteins in the formed protein corona changes [215,374,375]. Tenzer et al. showed that changing time resulted as significant changes in the corona composition even for 2 minute changes in the exposure time and early time points caused more significant changes when compared with longer time exposure [285]. Furthermore, Giau et al. also reported that when nanoparticles interacted with cell culture medium for 5 and 60 minutes, total of 14 proteins differ after 1-hour interaction when compared with 5-minute interaction and also abundancy of the some of the proteins increased and some of them decreased when time increased to 1 hour. Changed composition could be explained by changed protein adsorption over time. Because fast bonded proteins with low binding affinity and short residence time could be replaced by long affinity proteins in the case of also hard corona proteins [373,381]. Moreover, bounded proteins change their folding during adsorption and undergo structural rearrangements [285]. Thus, changed protein band intensities could be related with the enriched and decreased protein amounts, structural rearrangement of proteins and adsorption of new proteins overtime. When two different cell culture media (DMEM and DMEM:F12 1:1 (DF12)) were compared, different bands were observed in gel images and also intensity of the same bands in two cell culture media were found as changed which means that type and amount of proteins changed with cell culture media. It is an expected result, because there are several reports showing that with the change of cell culture media, PrC protein profile could change [374,377].

On the other hand changes in the FBS concentration (10 percent and 5 percent FBS) could also alter PrC on NMs [375,376] and as seen, when FBS concentration changes, intensities of the obtained bands changed in the case of AuNPs with 10 and 5 percent FBS included media. Consequently, it was found that PrC conformation changes by time due to its dynamic structure and it is dependent on cell culture media and FBS concentration.

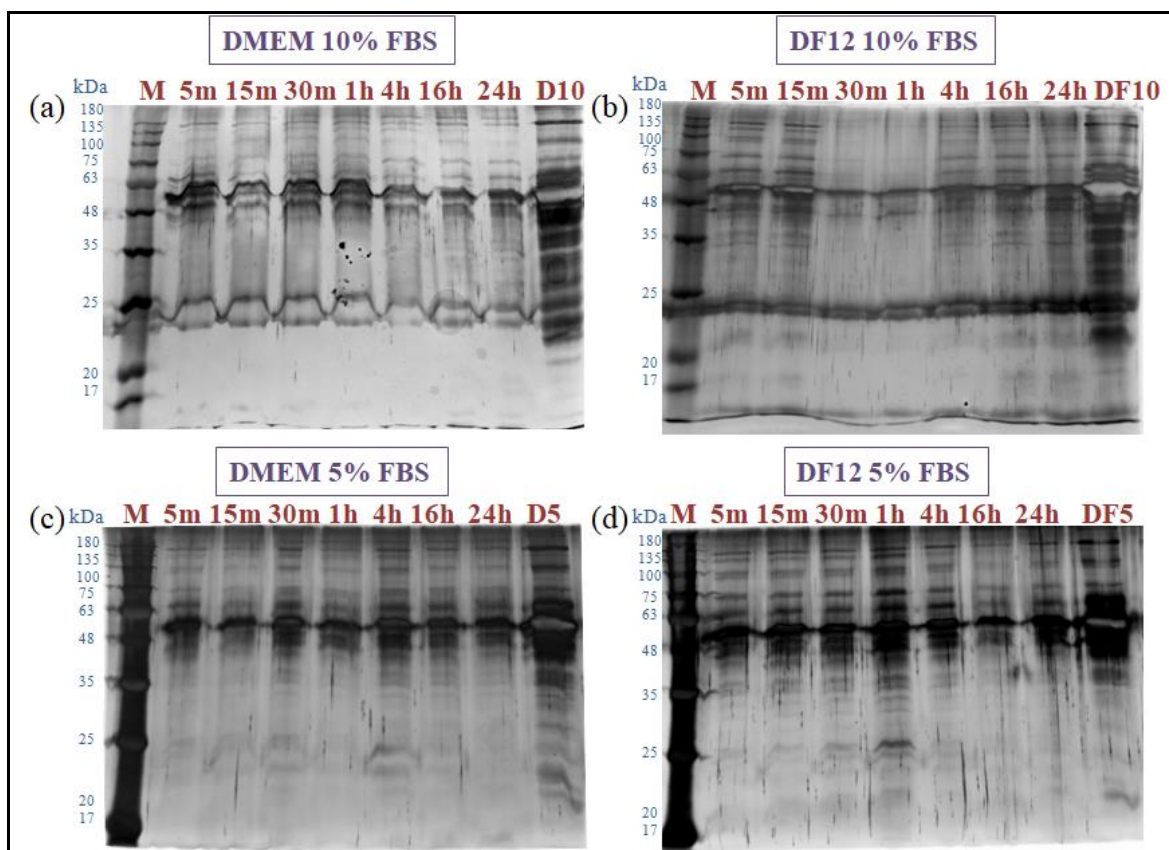


Figure 4.9. Silver stained gel images of AuNPs-protein corona samples formed in 5 min, 15 min, 30 min, 1 h, 4 h, 16 h and 24 h (a) DMEM with 10 percent FBS (D10), (b) DMEM-F12 Ham with 10 percent FBS (DF10), (c) DMEM with 5 percent FBS (D5), (d) DMEM-F12 Ham with 5 percent FBS (DF5). M: Marker

4.2.3. Cellular Uptake

In this study, not only PrC on the AuNPs was characterized but also affect of the PrC on the uptake of AuNPs were also examined. As seen in Figure 4.10.a, each cell internalizes different amount of AuNPs based on altered SSC shifts of the cells. On the other hand, when AuNPs were internalized by different cell culture media and different FBS

concentrations as seen in Figure 4.10.b, internalized amount of AuNPs changes for each cell line. For Beas-2b cell line, DMEM cell culture media with 10 percent FBS caused highest uptake and for A549 cell line, DMEM:F12 with 5 percent FBS, for PNT1A cell line, DMEM:F12 with 10 percent FBS and for HeLa, DMEM with 5 percent FBS caused highest FBS. Altered AuNPs uptake is related to the altered PrC structure with the use of different cell culture conditions and it is known that altered PrC structure directly changes toxicity, uptake, internalization pathway, or even clearance of the NMs [363,376,382,383].

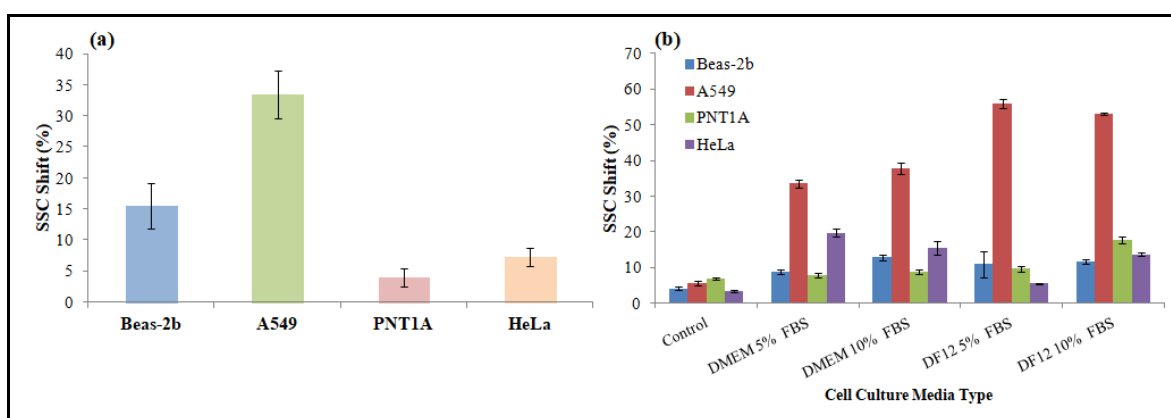


Figure 4.10. SSC Shift results of (a) Beas-2b, A549, PNT1A and HeLa cells treated with AuNPs in their own cell culture media (DMEM with 5 percent FBS for Beas-2b, DMEM-F12 Ham 1:1 with 10 percent FBS for A549, DMEM with 10 percent FBS for PNT1A and HeLa), (b) Beas-2b, A549, PNT1A and HeLa cells treated with AuNPs in DMEM with 5 percent FBS, DMEM with 10 percent FBS, DMEM-F12 Ham 1:1 (DF12) with 5 percent FBS, DMEM-F12 Ham 1:1 (DF12) with 10 percent FBS. Control SSC shifts were obtained by without AuNPs treatment for each cell in their own growing cell culture media (DMEM 5 percent FBS for Beas-2b, DF12 with 10 percent FBS for A549, DMEM 10 percent FBS for both PNT1A and HeLa cells)

After the determination of the internalized amount of AuNPs in different cell culture conditions, endocytosis pathway choice of AuNPs in different cell culture conditions were examined and the results are shown in Figure 4.11. As seen, each cell culture condition caused differential uptake of AuNPs by different SSC shifts for each cell line. Obtained results were summarized at Table 4.6.

For Beas-2b cell line, when its growing cell culture conditions DMEM with 5 percent FBS is used, AuNPs were internalized by all three endocytosis pathways as ClME, CaME and macropinocytosis. On the other hand, when different cell culture media conditions were used, Scr and Cpm blocked ClME only at DMEM with 5 percent FBS and DMEM:F12 (DF12) with 5 percent FBS. On the other hand, CaME was inhibited by Nys and Fil for DMEM with 10 percent FBS and DF12 with 5 percent FBS significantly. For macropinocytosis inhibition, controversial results were obtained for CytoD and Aml. CytoD inhibited macropinocytosis at all cell culture conditions while Aml inhibited only DMEM with 10 percent FBS and DF12 with 5 percent FBS. As a consequence, for DF12 with 10 percent FBS, internalization of least amount of AuNPs was inhibited and only macropinocytosis was found as internalization pathway. However, for DF12 with 5 percent FBS, all pathways were inhibited which means AuNPs were internalized by three pathways when FBS amount decreases for same cell culture media type. For DMEM with 10 percent FBS, CaME and macropinocytosis was found as internalization pathways of AuNPs while for DMEM with 5 percent FBS, ClME and macropinocytosis were used for uptake.

For A549 cell line, it was found that like Beas-2b all three endocytosis pathways were used for the uptake of AuNPs when DF12 with 10 percent FBS was used as growing cell culture conditions. On the other hand, when conditions were changed, it was found that only Scr could decrease the internalized amount of AuNPs for DMEM with 5, 10 percent FBS and DF12 with 5 percent FBS significantly although with CPM treatment no significant decrease was achieved for ClME. For CaME inhibition, both Nys and Fil decreased AuNPs amount for all of the cell culture conditions with different decreased amount of AuNPs. When macropinocytosis was considered, CytoD and Aml inhibited AuNPs uptake with DMEM 5, 10 percent FBS and DF12 with 5 percent FBS. Consequently, when DMEM with 5 FBS, DMEM with 10 percent FBS and DF12 with 5 percent FBS was used, AuNPs were uptaken by all three pathways while when DF12 with 10 percent FBS was used CaME and ClME was used for the uptake of AuNPs.

For HeLa cell line, when growing cell culture conditions DMEM with 10 percent FBS was used, AuNPs were internalized by only CaME. However, when DMEM used with 5 percent FBS, ClME was also inhibited. When cell culture medium is changed as DF12

which is used with 5 or 10 percent FBS, not only ClME and CaME but also macropinocytosis was inhibited.

For PNT1A cell line, ClME CaME and macropinocytosis were used for AuNPs uptake when growing medium DMEM with 10 percent FBS was used. When DMEM is used with 5 percent FBS, all three endocytosis pathways were found as involved in AuNPs uptake but with different uptaken rates. On the other hand, when cell culture media is changed as DF12, significant decrease in the AuNPs uptake could not observed in any endocytosis pathways and only decrease was observed at Scr for DF12 with 10 percent. Consequently, it could be said that, endocytosis of AuNPs is not only cell type but also PrC related parameters cell culture media and FBS concentration dependent.

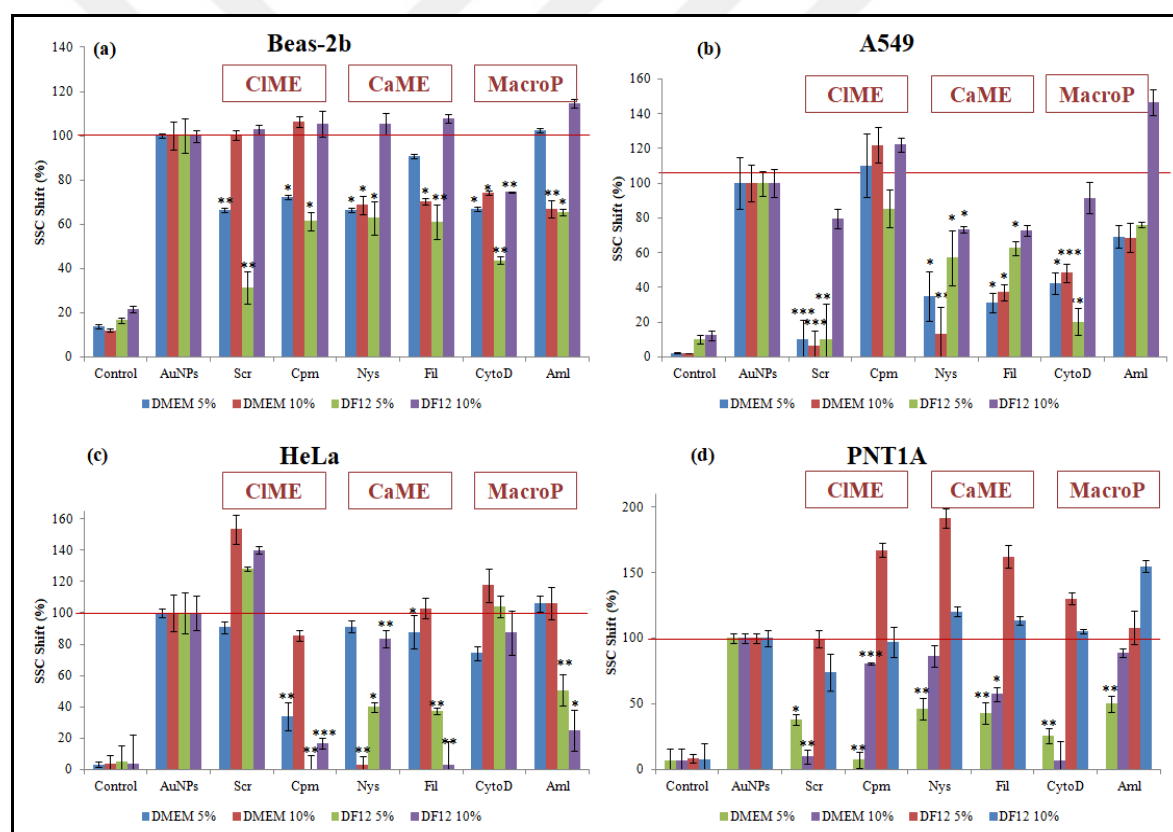


Figure 4.11. Uptake of (a) Beas-2b, (b) A549, (c) HeLa and (d) PNT1A cells in different cell culture media in the presence of endocytosis inhibitors. ClME: Clathrin mediated endocytosis, CaME: Caveolae mediated endocytosis. MacroP: Macropinocytosis: Scr: Sucrose, Cpm: Chlorpromazine, Nys: Nystatin, Fil: Filipin, CytoD: Cytochalasin D, Aml: Amiloride

Table 4.6. Summary of AuNPs internalization pathway choice with different cell culture conditions

Beas-2b		Scr	Cpm	Nys	Fil	CytoD	Aml
D5%		+	+	+	-	+	-
D10%		-	-	+	+	+	+
DF5%		+	+	+	+	+	+
DF10%		-	-	-	-	+	-

A549		Scr	Cpm	Nys	Fil	CytoD	Aml
D5%		+	-	+	+	+	+
D10%		+	-	+	+	+	+
DF5%		+	-	+	+	+	+
DF10%		+	-	+	+	+	-

HeLa		Scr	Cpm	Nys	Fil	CytoD	Aml
D5%		-	+	-	+	-	-
D10%		-	-	+	-	-	-
DF5%		-	+	+	+	-	+
DF10%		-	+	+	+	-	+

PNT1A		Scr	Cpm	Nys	Fil	CytoD	Aml
D5%		+	+	+	+	+	+
D10%		+	+	-	+	+	-
DF5%		-	-	-	-	-	-
DF10%		-	-	-	-	-	-

4.2.4. SERS Measurements

The protein corona formation has two separated stages which is explained with "Vroman effect" [384]. At the first stage, when NPs are added into the biological environment, they interact with biomolecules possessing greater affinity to the NP surfaces and initial corona is formed. At the second stage; competition of proteins lead the changes in the corona composition and proteins with short residence time is replaced by the ones with slower association rates and longer residence time [385]. Thus, even starting from the time when

NPs added to the environment; formed corona composition changes. When protein corona formation is investigated *ex situ*, soft corona formation and corona composition change can be examined. Thus, SC formation was investigated by SERS as seen in Figure 4.12. A list of the tentative peak assignments is given in Table 4.7. In 4 different cell culture media, SC formation was tracked overtime and it was found that SC changes overtime causing spectral changes in the SERS spectra not only time dependent but also media and FBS concentration dependent.

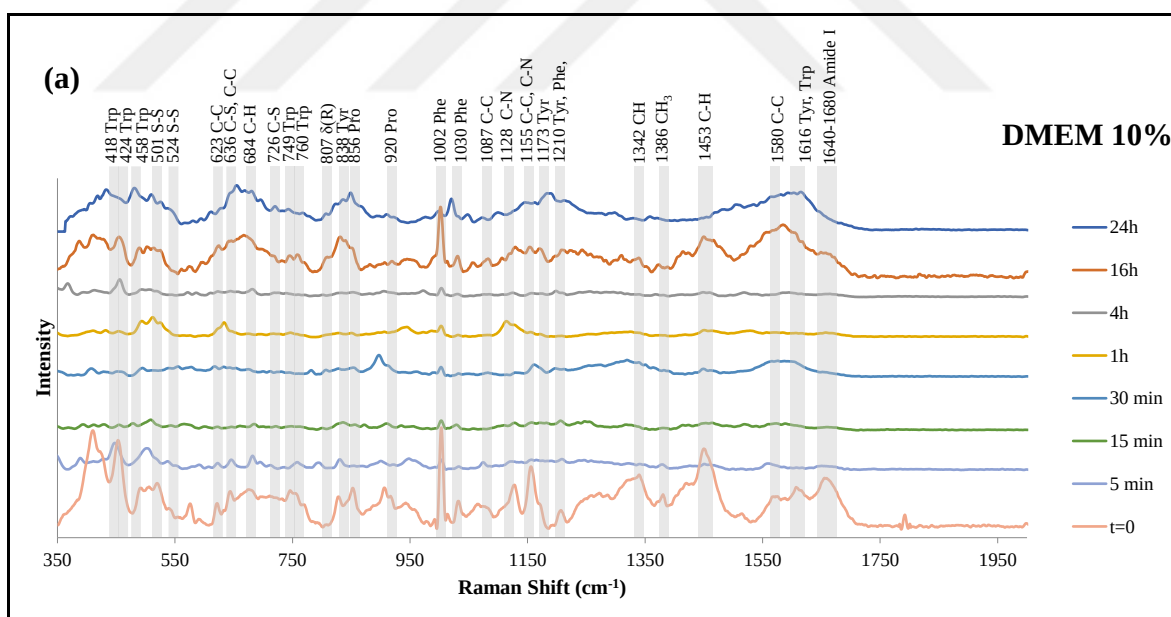
As shown in Figure 4.12., SC changes as time dependent manner when AuNPs were treated with 4 different cell culture conditions. It was found that except for the first interaction between media and AuNPs at $t=0$, band intensities increase over time for all conditions. For 4 different conditions, $t=0$ shows totally different SERS pattern due to the first interaction of proteins with AuNPs surface which is explained by Vroman effect. In the first interaction, high mobility and low molecular weighted proteins come close vicinity to the NPs surface while with increasing time these proteins replace by more stable, high molecular weighted proteins with higher binding affinity [386]. Thus, this change was reflected by the SERS spectra as change of pattern overtime. The spectral bands related to the protein structures were detected at different time points. At $t=0$, different spectral bands with high intensity were detected. Most of the detected bands were related to the amino acids including tryptophan (Trp) (418 cm^{-1} , 424 cm^{-1} , 458 cm^{-1} , 749 cm^{-1} , 760 cm^{-1}), tyrosine (Tyr) (646 cm^{-1} , 838 cm^{-1} , 1173 cm^{-1} , 1616 cm^{-1}), phenylalanine (Phe) (1002 cm^{-1} , 1030 cm^{-1} , 1210 cm^{-1}) and proline (Pro) (920 cm^{-1}). Molecular band vibrations were also detected including disulfide bands (501 cm^{-1} , 524 cm^{-1}), C-C (623 cm^{-1} , 646 cm^{-1}), C-S (636 cm^{-1}), C-H (684 cm^{-1} , 1173 cm^{-1}) bands and structural bands including Amide I (1640 cm^{-1}) and Amide II (1558 cm^{-1}). For the spectral bands attributed to the amino acids, the intensity of the observed bands was found as decreased overtime up to 4 h and then increased with increasing time, which could be related with the dissociation of high mobility and less affinity proteins from the surface and replaced with higher affinity proteins. These intensity changes could be correlated with the other results. The first interaction between AuNPs and proteins in the cell culture media caused sudden hydrodynamic size increase up to 80 nm and zeta potential decrease up to -6.5 m. Up to 1 h, the hydrodynamic size decreased and after 1 h, the size increased with increasing time. On the other hand, zeta potential was increased after $t=0$ overtime. According to these

results, up to 1 h, thinner protein layer was formed on the AuNPs and then layer thickness increased. When the composition is examined by SDS-PAGE, it was found that by changing time, abundance of the proteins changes by changed band intensities. Thus, when AuNPs interacted with the PrC for shorter time after the first interaction, formed protein layer was smaller and interaction of proteins in that layer did not provide intense SERS peaks in the obtained spectra. Besides, peaks at 424 cm^{-1} (Trp), 501 cm^{-1} (S-S), 623 cm^{-1} (C-C), 636 cm^{-1} (C-C, C-S), 684 cm^{-1} (C-H), 749 cm^{-1} (Trp), 760 cm^{-1} (Trp), 838 cm^{-1} (Trp), 1002 cm^{-1} (Phe), 1128 cm^{-1} (C-N), 1155 cm^{-1} (C-C, C-N), 1210 cm^{-1} (Tyr, Phe), 1580 cm^{-1} (C-C) were observed in the SERS spectra when AuNPs were interacted with PrC less than 1 h. After 1 h treatment, size of the AuNPs increased and protein profile was found as changed and these changes reflected to the SERS spectra as new and more intense bands at 1616 cm^{-1} (Tyr, Trp), 1173 cm^{-1} (Tyr, Phe), 1087 cm^{-1} (C-C) at both 16 and 24 h. On the other hand, peaks at 1680 cm^{-1} (Amide I), 1640 cm^{-1} (Amide I), 1453 cm^{-1} (Proteins), 1342 cm^{-1} (C-H), 920 cm^{-1} (Pro) were observed only 16 h while peaks at 726 cm^{-1} (C-S), 684 cm^{-1} (C-H, COO⁻), 458 cm^{-1} (Trp) were observed at only 24 h. These data show that changes in the protein corona structure can be detected by SERS and time dependent changes in the protein corona composition can be tracked by specific SERS spectral bands.

When AuNPs were interacted with DMEM-F12 (DF12) included 10 percent FBS as shown in Figure 4.12.b, similar spectral pattern was observed with DMEM included 10 percent FBS. Like in the DMEM 10 percent FBS, $t=0$ provided intense SERS signals while these signals decreased after first interaction up to 4 h and increased after 4 h treatment. Even pattern of the obtained SERS signal is similar; it is known that using different cell culture media with different compounds and concentrations provide differences in the protein corona structure. When compared to the other cell culture conditions, DF12 provided different protein profile and smaller hydrodynamic size increase with broader SPR shift than DMEM type of cell culture media. Using 10 percent FBS caused slightly higher SPR shift, bigger hydrodynamic size increase and zeta potential change with increasing time. Moreover, obtained intensity changes were smaller than DMEM type of cell culture media and intensity changes at 1210 cm^{-1} (Tyr, Phe), 920 cm^{-1} (Pro), 807 cm^{-1} (Ring deformation) and 636 cm^{-1} (C-S, C-C) was not observed with DF12 10 percent FBS. On the other hand,

intensity changes at 1470 cm^{-1} (C=N) and 646 cm^{-1} (Tyr) were observed at $t=0$, 16 h and 24 h treatment.

When FBS concentrations were compared, both DMEM with 5 percent FBS and DF12 with 5 percent FBS type of media composition provided higher signal intensities when compared with 10 percent FBS concentration. It could be caused by the concentrations of proteins in the serum and when the protein amount is much, it could suppress the signal because as seen in Figure 4.13., without any AuNPs, media did not have SERS peak but still have a high signal rate which could suppress the coming signal when concentration increases. For the 5 percent FBS concentration, both type of the cell culture media provided similar SERS signal while slight differences based on the media was observed. When DMEM was used as cell culture media, peak intensity at 623 cm^{-1} (C-C) were observed similarly with DF12. When DMEM-F12 was used as cell culture media, new peaks at 1558 cm^{-1} (Trp) and 920 cm^{-1} (Pro) were observed. These slight differences were observed due to the altered protein corona composition based on media type.



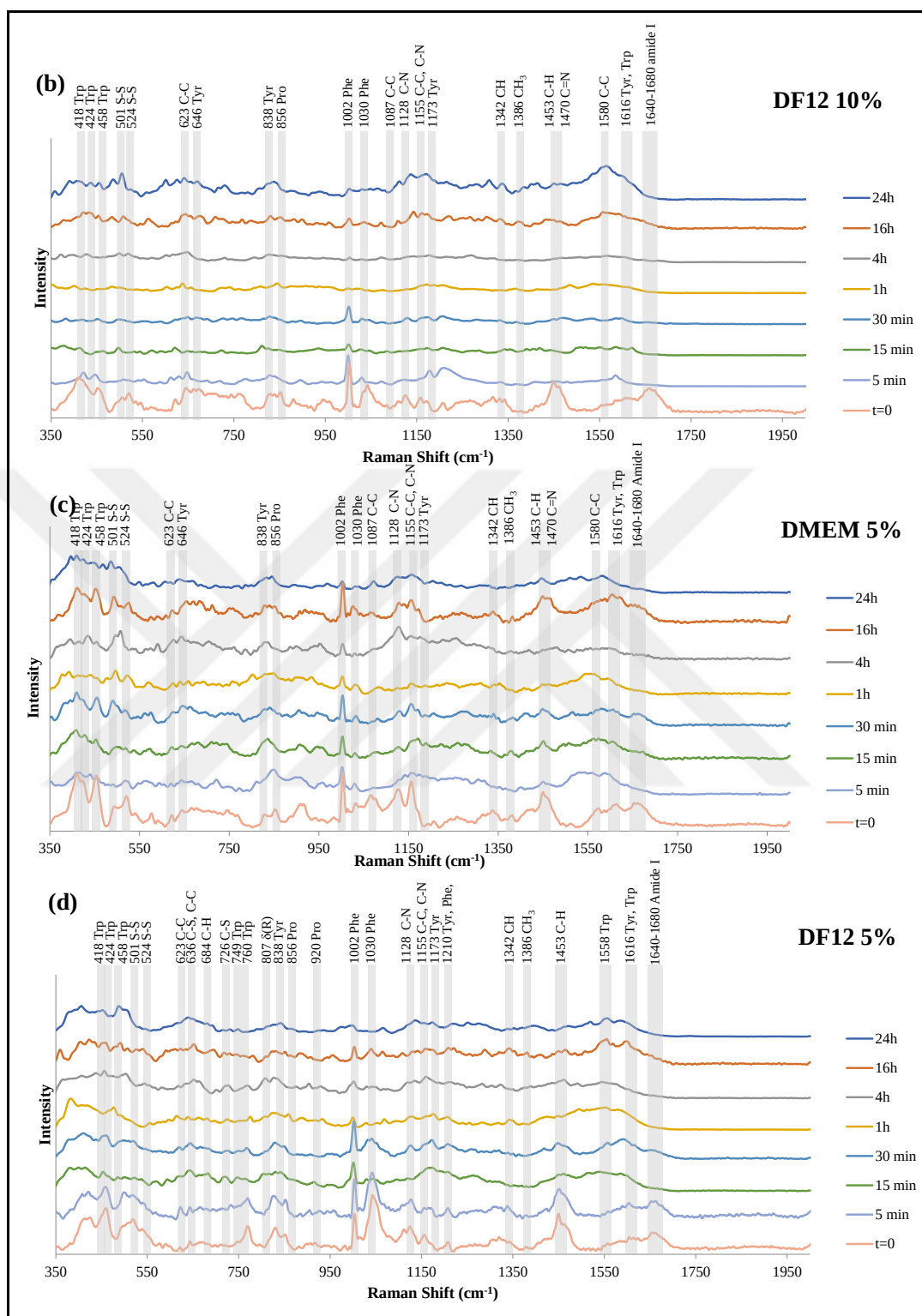


Figure 4.12. Average SERS spectra of AuNPs without any centrifugation (SC) in (a) DMEM with 10 percent FBS, (b) DMEM:F12 with 10 percent FBS, (c) DMEM with 5 percent FBS and (d) (b) DMEM:F12 with 10 percent FBS. ν : stretching, δ : deformation, Trp: tryptophan, Tyr: tyrosine, Pro: proline, Phe: phenylalanine

Table 4.7. Tentative SERS Band Assignments. Trp: tryptophan, Tyr: tyrosine, Pro: proline, Phe: phenylalanine

cm⁻¹	Assignment
418	Trp
424	Trp
458	Trp: C ₆ , C ₅ ring deformation
501	S-S gauche-gauche-gauche stretching
524	S-S disulfide stretching in proteins
623	C-C twist aromatic ring
636	C-S stretching & C-C twisting (Proteins)
646	C-C twisting mode of tyrosine
684	C-H, COO ⁻ , ring deformation
726	C-S (Protein)
749	Symmetric breathing of Trp (Protein assignment)
760	Ring breathing Trp (Proteins)
807	C ₆ ring deformation
838	Ring breathing (Tyr)
856	Amino acid side chain vibrations of proline
920	C-C stretch of Pro ring
1002	Phenylalanine
1030	Phenylalanine, C-N stretching (Proteins)
1087	C-C stretching
1128	C-N stretching (Proteins)
1155	C-C and C-N stretching of proteins
1173	Tyr, Phe, C-H bend (Proteins)
1210	C-C ₆ H ₅ stretching mode in Tyr and Phe
1342	CH deformation
1386	CH ₃ band
1453	Proteins
1470	C=N stretching
1558	Tryptophan, C-N stretching and N-H deformation (Amide II)
1580	C-C stretching
1616	C=C stretching mode of tyrosine & tryptophan
1640	Amide I

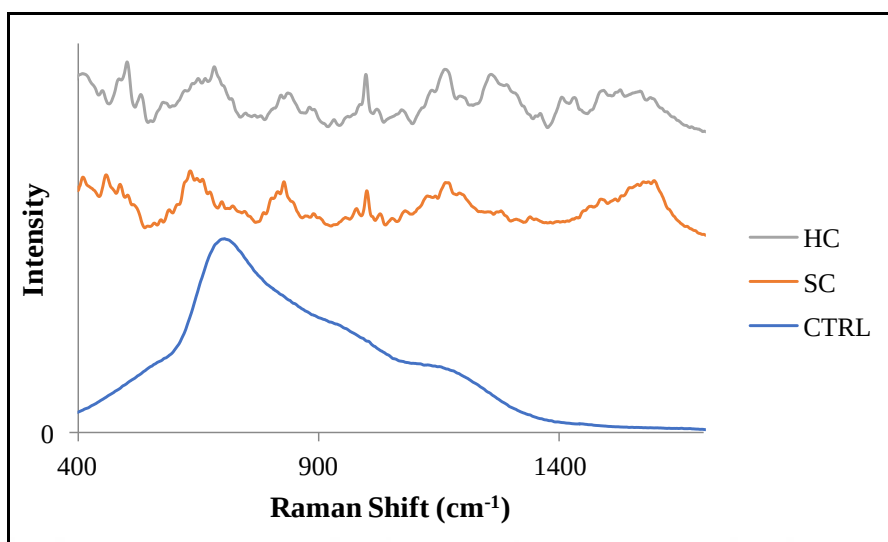


Figure 4.13. Comparison of SERS spectra of cell culture media (CTRL), Soft corona (SC) and Hard Corona (HC) on the AuNPs

After the investigation of SC on the AuNPs surface by SERS, HC on the AuNPs surface was examined by removing low affinity proteins from the surface by centrifugation and obtained SERS spectra is shown in Figure 4.14. As seen, after centrifugation almost all spectral bands were changes and more peaks were observed with the contribution of new spectral bands. Spectral bands attributed to amino acids including tryptophan (418 cm^{-1} , 743 cm^{-1} , 770 cm^{-1} , 1365 cm^{-1} , 1558 cm^{-1}), tyrosine (653 cm^{-1} , 838 cm^{-1} , 1173 cm^{-1}), phenylalanine (1002 cm^{-1} , 1030 cm^{-1} , 1586 cm^{-1}) and structural bands including C-C (623 cm^{-1} , 653 cm^{-1} , 938 cm^{-1}), C-S (449 cm^{-1} , 636 cm^{-1}), C-H (684 cm^{-1} , 1173 cm^{-1} , 1449 cm^{-1}), C-N (1083 cm^{-1} , 1099 cm^{-1}), C=N (1470 cm^{-1}), secondary structure including Amide I (1602 cm^{-1}), Amide II (1565 cm^{-1} , 1544 cm^{-1}) and Amide III (1317 cm^{-1} , 1290 cm^{-1} , 1279 cm^{-1} , 1260 cm^{-1} , 1250 cm^{-1} , 1224 cm^{-1} , 1204 cm^{-1}) was observed. When obtained results were examined overtime, similar patterns were observed. Compared with SC measurements, similar results were achieved with different time points and this could be the results of the centrifugation and elimination of different SC protein associations with different time interactions. In each time points, when PrC-AuNPs complex is centrifuged, SC is removed and HC remains. Due to having high affinity and longer surface residence time, changes in the HC composition would be smaller and provide similar spectral pattern in the SERS measurement. Although in each time points, observed spectral pattern is found as similar, time dependent changes were also observed in each cell culture condition.

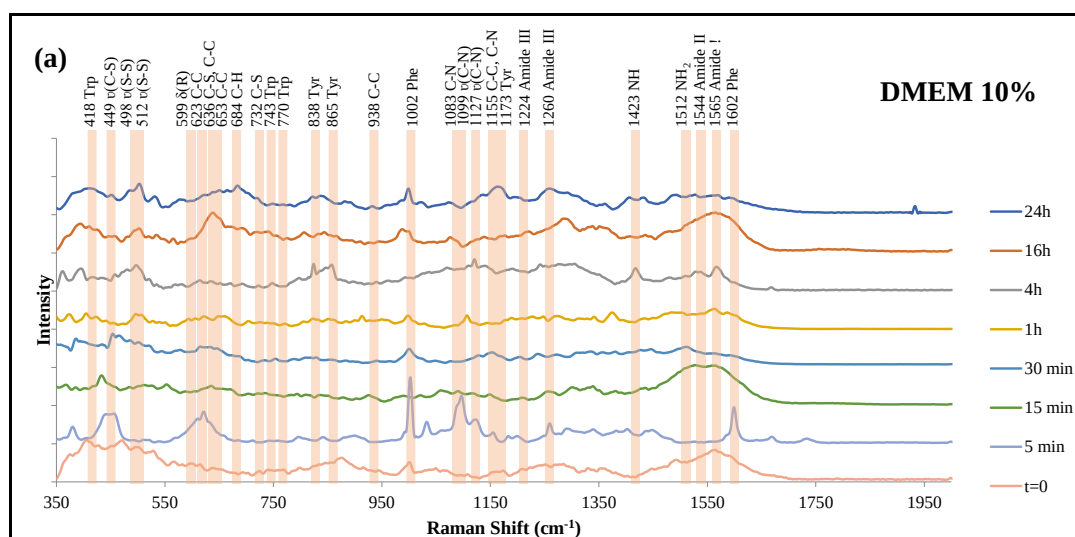
When AuNPs is interacted with DMEM including 10 percent FBS, 5 min interaction provided best signals including spectral bands at 1602 cm^{-1} (Phe), 1260 cm^{-1} (Amide I), 1155 cm^{-1} (C-C, C-N), 1127 cm^{-1} (C-N), 1099 cm^{-1} (C-N), 1002 cm^{-1} (Phe), 623 cm^{-1} (C-C), 449 cm^{-1} (C-S). Most of these bands lose their intensity with increasing time and leave their places to the new peaks at 1565 cm^{-1} (Amide I), 1512 cm^{-1} (NH_2), 1423 cm^{-1} (NH), 1224 cm^{-1} (Amide III), 1083 cm^{-1} (C-N), 865 cm^{-1} , 838 cm^{-1} (Tyr), 770 cm^{-1} , 743 cm^{-1} (Phe). These changes should be the results of association and dissociation of proteins found in the HC complex and which was also observed as increasing hydrodynamic size decreased zeta potential change.

When AuNPs interacted with DF12 including 10 percent FBS, different peaks at 1204 cm^{-1} (Amide III), 1030 cm^{-1} (Phe) and 890 cm^{-1} (Proteins) were observed when compared with DMEM and intensities of the obtained bands were increased after 4 h treatment. After 4 h treatment it was found that hydrodynamic size of the AuNPs increase up to 95 nm, zeta potential increases up to -26 mV, and SPR shift slightly increase and intensity of protein bands in the SDS-PAGE. These results provide that the HC structure changes and these changes could be tracked by SERS which is reflected as increased band intensity for some of the spectral bands and also observation of new bands with increasing time such as 1586 cm^{-1} (Ring deformation), 1224 cm^{-1} (Amide III), 838 cm^{-1} (Tyr), 626 cm^{-1} (C-C), 498 cm^{-1} (S-S), 418 cm^{-1} (Trp).

Similar to the SC results, when FBS concentration decreased to 5 percent in the media, better SERS signal was achieved for both DMEM and DF12 type of cell culture media. When two different media were compared for 5 percent FBS, although DMEM type media provided more signal when compared with DF12, 5 percent FBS concentration provided same peak observations in two different media including peaks at 1558 cm^{-1} (Trp), 1506 cm^{-1} ($\text{N}=\text{H}$), 1470 cm^{-1} ($\text{C}=\text{N}$), 1155 cm^{-1} (C-C, C-N), 1002 cm^{-1} (Phe), 938 cm^{-1} (C-C), 838 cm^{-1} (Tyr), 743 cm^{-1} (C-H, C-S), 732 cm^{-1} (COO^- , C-S), 653 cm^{-1} (Tyr), 636 cm^{-1} (C-S, C-C), 623 cm^{-1} (C-C). On the other hand, DMEM type media provided different peaks attributed to structural bands and secondary structure were observed. For DF12 different peaks at 1343 cm^{-1} (C-H), 1308 cm^{-1} (C-N), 1290 cm^{-1} (Amide III), 1250 cm^{-1} (Amide III) were observed. These similarities and slight differences could be coming from using same FBS concentration but different cell culture media. Using same amount of proteins could

result as the adsorption of proteins with similar longer surface residence time while using different media type could result as the changes in the corona conformation or abundances.

When results of HC and SC were compared, some of the SERS peaks were found similar in the obtained spectra. For instance, peaks at 1155 cm^{-1} , 1030 cm^{-1} , 1002 cm^{-1} , 838 cm^{-1} , 636 cm^{-1} , 623 cm^{-1} , 501 cm^{-1} and 418 cm^{-1} were observed not only in the SERS spectra of SC-AuNPs complex but also HC-AuNPs complex. Interestingly, when obtained SERS spectra from PrC complex was compared with intracellular SERS spectra, except for 418 cm^{-1} , remaining peaks were also observed at intracellular spectra as shown in Figure 4.15. These results show direct interfere of the PrC on the AuNPs surface in the obtained intracellular SERS spectra. It was known that even SC dissociates during the internalization of nanoparticles, HC remains and nanoparticle continues its journey as HC-AuNPs complex [217-219]. Moreover, it should be also noted that observation of mentioned peaks at both SC and HC could be the results of the proteins belong to the HC which did not dissociate after centrifugation and stay still on the AuNPs. When proteins did not dissociate with centrifuge and remain during endocytosis, they will contribute to the obtained SERS spectra because they will be still close vicinity to the AuNPs surface. Thus, obtained peak observation or changes at 1155 cm^{-1} , 1030 cm^{-1} , 1002 cm^{-1} , 838 cm^{-1} , 636 cm^{-1} , 623 cm^{-1} , 501 cm^{-1} in the intracellular spectra could be affected by the PrC formation on the AuNPs. Consequently, when a cellular SERS spectrum is considered, it should be always thought that PrC could contribute to the obtained results whose composition could alter with using not only different concentration of protein source but also different cell culture media type.



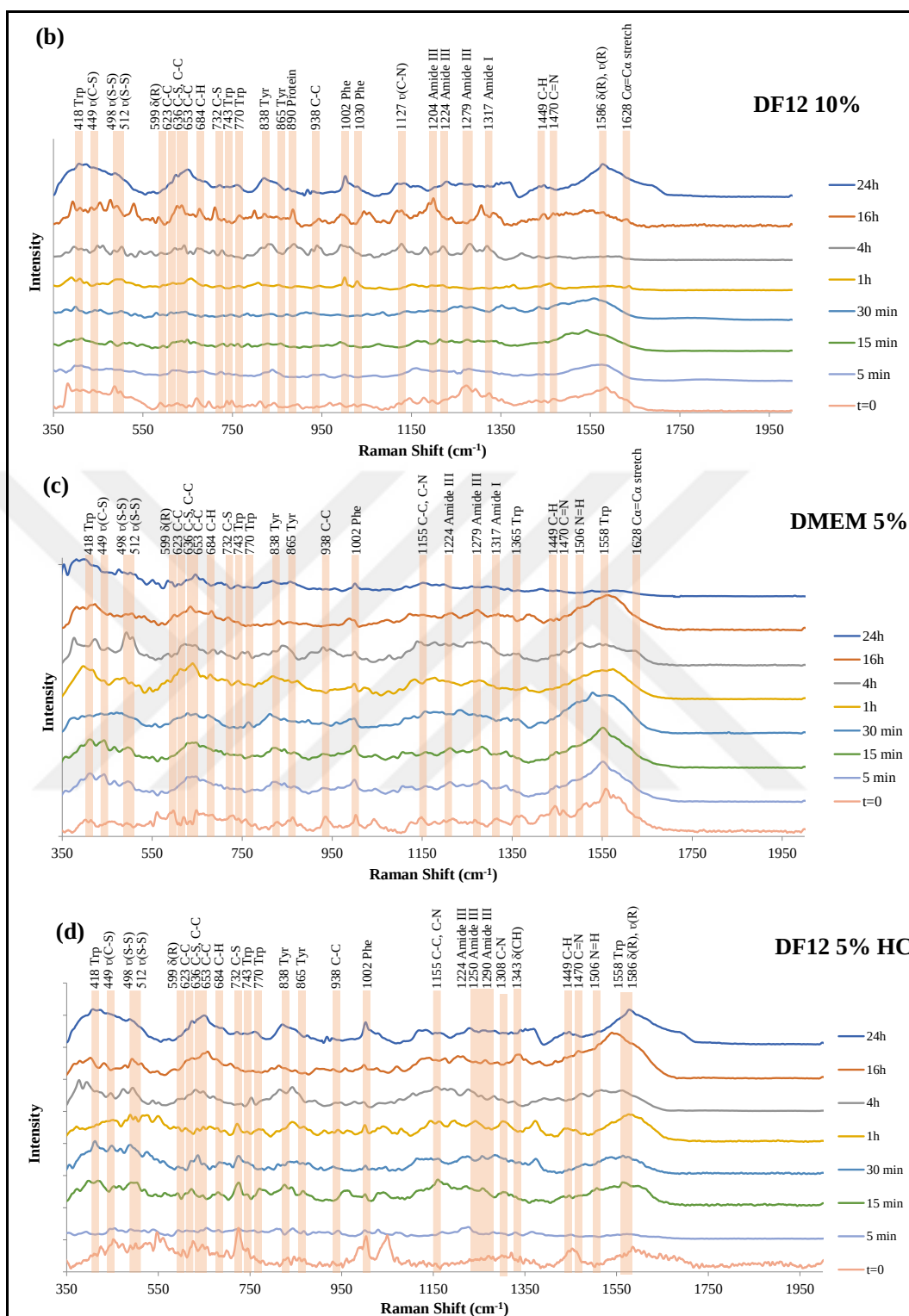


Figure 4.14. Average SERS spectra of AuNPs after centrifugation (HC) in (a) DMEM with 10 percent FBS, (b) DMEM:F12 (DF12) with 10 percent FBS, (c) DMEM with 5 percent FBS and (d) DMEM:F12 with 10 percent FBS. ν : stretching, δ : deformation, Trp: tryptophan, Tyr: tyrosine, Pro: proline, Phe: phenylalanine

Table 4.8. Tentative SERS Band Assignments. Trp: tryptophan, Tyr: tyrosine, Pro: proline, Phe: phenylalanine

cm⁻¹	Assignment
418	Trp
449	C-S stretching
498	S-S stretching
512	S-S stretching
599	Ring deformation
623	C-C twist aromatic ring
636	C-S stretching & C-C twisting (Proteins)
653	C-C twisting (Tyrosine)
684	C-H, COO ⁻ , ring deformation
732	COO ⁻ deformation, C-S stretching
743	C-H deformation, C-S stretching, Trp
770	Trp
838	Ring breathing (Tyr)
865	Tyr, C-CH deformation
890	Proteins
938	C-C stretching
1002	Phenylalanine
1030	Phenylalanine, C-N stretching (Proteins)
1083	C-N stretching mode of proteins
1099	C-N stretching
1155	C-C (and C-N) stretching (Proteins)
1173	Tyr, Phe, C-H bend (Proteins)
1204	Amide III
1224	Amide III (β sheet structure)
1250	Amide III
1260	Amide III
1279	Amide III (α -helix)
1290	Amide III, CH ₂ and CH ₃ deformation, CH bending
1308	C-N asymmetric stretching
1317	Amide III (α -helix)
1343	C-H deformation
1365	Trp
1423	NH in-plane deformation
1449	C-H vibration (Proteins)
1470	C=N stretching
1506	N=H bending
1512	NH ₂ bend, ring stretching
1544	Amide II
1558	Trp
1565	Amide II
1586	Phe
1602	C=O stretching (Amide I)
1628	C α =C α stretch

After the comparison of the SERS spectra of the PrC with intracellular spectra, cellular spectra changes based on different protein corona was also examined as cell type dependent manner and obtained results are shown in Figure 4.15. with tentative band assignments for intracellular SERS spectra in Table 4.9. When 4 different cell lines were treated with PC-AuNPs complex using 2 different cell culture media and 2 different FBS concentration, different spectral intensities were observed for each cell line. In all cell lines, DF12 with 5 percent FBS provided lower intensity and HeLa cell line was found as most affected cell line based on the use of different cell culture conditions.

For Beas-2b cell line, DF12 with 10 percent FBS, DMEM with 5 and 10 percent FBS provided similar spectral pattern while using DMEM with 5 percent FBS as cell culture condition caused significant differences in the obtained SERS spectra. With the use of DMEM with 5 percent FBS, intensities of the peaks corresponding to proteins (1317 cm^{-1} , 1199 cm^{-1} , 1155 cm^{-1} , 1002 cm^{-1} , 573 cm^{-1}) and phospholipid at 1130 cm^{-1} significantly differed from any other type of cell culture composition. These significant changes are the result of the altered PC structure which altered uptake amount and route of AuNPs into the cells. For Beas-2b cell line, although uptake amount of AuNPs is similar between cell culture conditions as seen in Figure 4.10., altered PrC composition changes uptake route of AuNPs as seen in Figure 4.11. which is reflected in the obtained SERS spectra. When DMEM with 5 percent FBS was used, Beas-2b cells internalized AuNPs using only micropinocytosis while in other cell culture conditions more than one endocytosis pathway is used for uptake. With the use of only one endocytosis pathway, surrounding environment of the AuNPs is restricted only by micropinocytosis whose molecules provide SERS spectra when they come close vicinity to the AuNPs surface. Due to having at least two different endosomal pathways with two different microenvironments, other type of cell culture conditions provided higher band intensities corresponding to the proteins and phospholipids which are reflected from two different pathways.

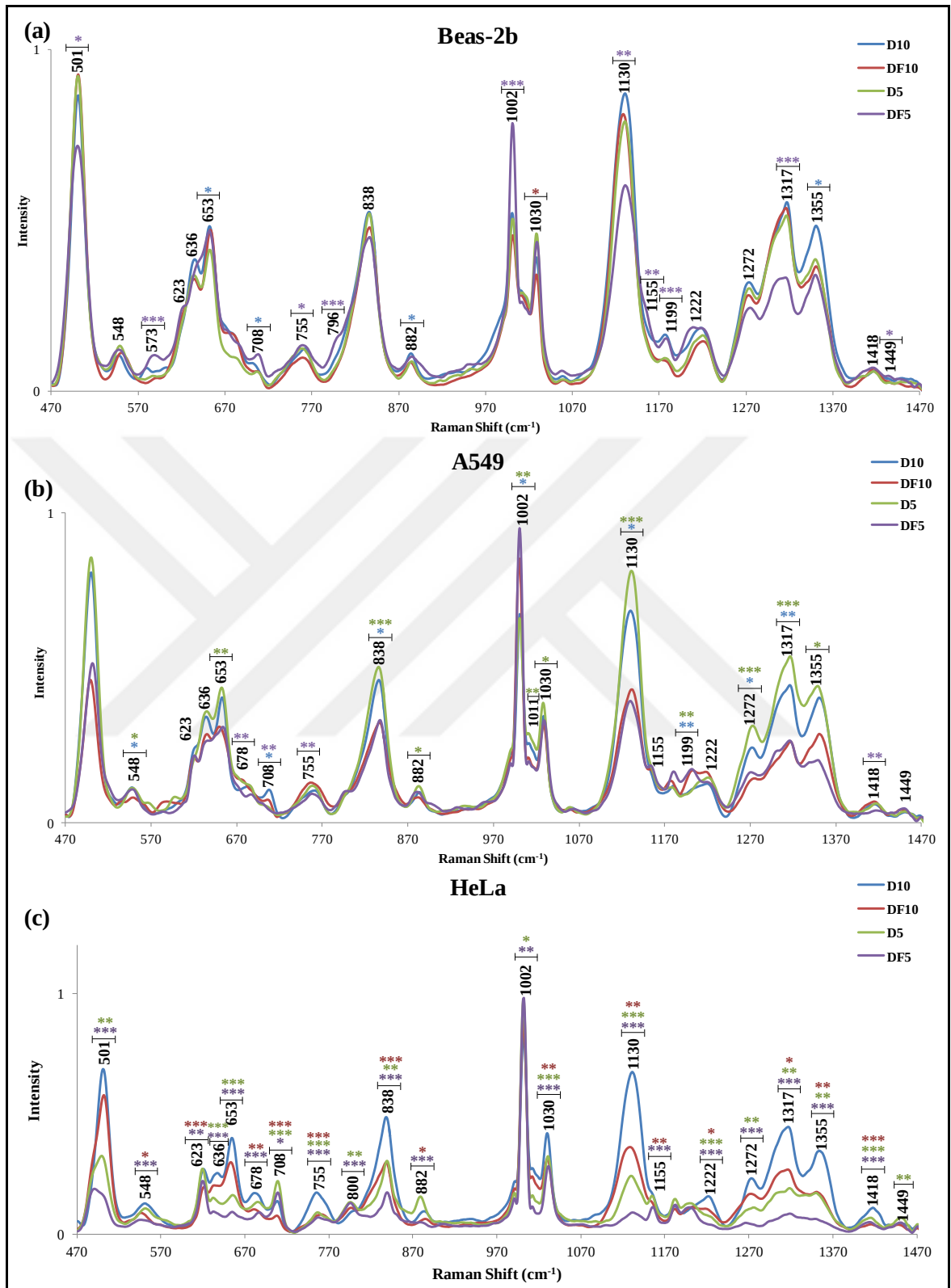
For A549 cell line, DMEM with 5 percent FBS provided highest signal and 10 percent FBS with DMEM (D10) and DF12 (DF10) cell culture media type provided least peak intensity. Even DF10 is the growth medium of A549 cell line and provided higher AuNPs uptake than both DMEM type cell culture conditions, it could not provide highest SERS

signal because of the endocytosis pathway differences of different cell culture conditions. Even DF12 with 5 percent FBS provided highest AuNPs uptake rate, it provided AuNPs internalization only by CaME while other cell culture conditions provided internalization with 3 different pathways. Using 3 different pathway provided AuNPs 3 different microenvironment whose signals were reflected in the SERS spectra as enhanced band intensities. It should be also noted that, using three different pathways did not provide different spectral pattern but provide enhanced band intensities because even endosomal pathways were considered as different pathways due to having distinct properties and molecular involvement during endosomal pathway development, they are all endocytosis pathways whose endosomal structures and molecular steps are similar when whole pathway is considered. When one pathway did not inhibit directly, they could provide similar spectral patterns obtained by spectral bands corresponding to mostly proteins, phosphatidylinositols and phospholipids. Thus, with the involvement only CaME, DF10 provided least signal.

For HeLa cell line, growth medium of HeLa cell line which is D10 provided highest signal while obtained signal intensities decrease with the use of DF10, D5 and DF5, respectively. When uptake amounts of AuNPs were considered, D10, DF10 and D5 provided similar uptake amounts which are higher than DF5. Obtained least signal by DF5 could be the result of the not enough AuNPs inside the cells and endosomes to get SERS signal. For remaining conditions, pathway dependent changes should be considered. When uptake amounts were similar, use of different endosomal pathway provided different spectral changes as seen in Beas-2b while different AuNPs uptake rate caused totally different response when cell type is changed as HeLa. With the use of D10, only CaME is used for internalization while with the use of DF10 and D5, three different pathways were used for the internalization. Observing higher peak intensities from just ClME could be the results of presence of the highest amount of AuNPs in the same endosomes formed by ClME which provided better signal enhancement when more AuNPs come closer inside the endosomes.

For PNT1A cell line, DMEM with 5 percent FBS provided similar signal intensities with DMEM-F12 with 10 percent FBS and the opposite was observed for DMEM with 10 percent FBs and DMEM-F12 with 5 percent FBS. For PNT1A cells, highest AuNPs uptake rate provided highest peak intensities for D5 and DF10 cell culture conditions but this

explanation could not be used for D10 type of media because even D10 provided higher uptake rate of AuNPs than DF5, they provided similar peak intensities at the SERS spectra. When uptake routes of AuNPs were considered, inhibitors could not provide any inhibition for DF5 which could be resulted by the internalization of very small amount of AuNPs. On the other hand, even D10 type of media resulted as the use of 3 different endosomal pathways like D5 type of media; it could not provide similar results with D5. This could be the results of the differences in the AuNPs uptake amount. Consequently, when all intracellular SERS spectra is examined as cell dependent manner, it was revealed that, obtained SERS spectra is cell dependent firstly and responses of the cells to the different conditions are directly reflected on the SERS spectra. When PrC structure on the AuNPs is altered by changing cell culture conditions, these alterations directly contribute to the obtained SERS spectra. It could be concluded as that PrC is one of the main parameters directly affecting SERS signal as cell type dependent manner.



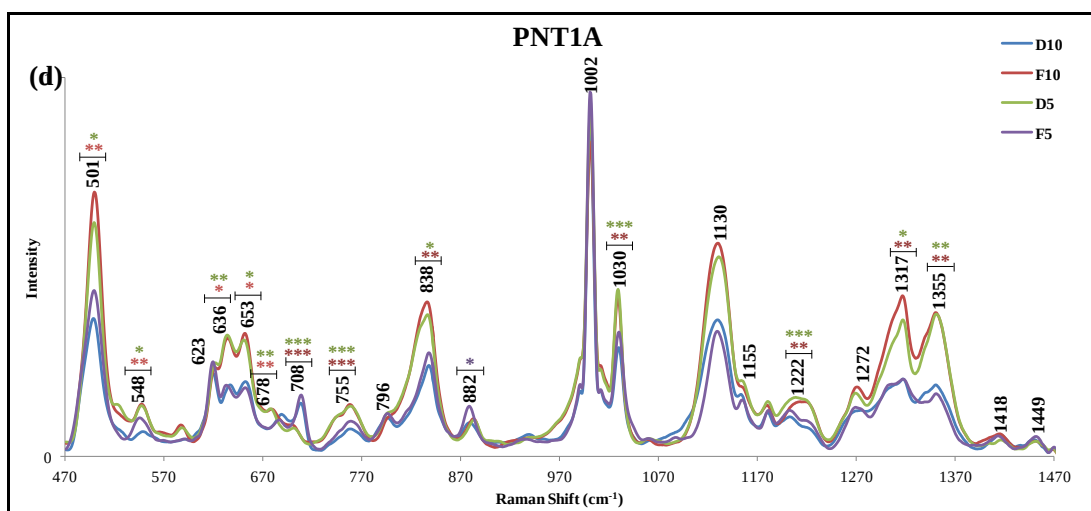


Figure 4.15. Average SERS spectra of (a) Beas-2b, (b) A549, (c) HeLa, and (d) PNT1A cells treated AuNPs in DMEM with 10 percent FBS (D10), DMEM:F12 with 10 percent FBS (DF10), DMEM with 5 percent FBS (D5), DMEM:F12 with 5 percent FBS (DF5). Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$ with the comparison of 2 μM Fil and control group. Each spectrum is compared to cell growth media where DMEM with 5 percent FBS for Beas-2b, DMEM-F12 with 10 percent for A549 and DMEM with 10 percent FBS for HeLa and PNT1A. Blue stars indicate t test comparison with DMEM 10 percent FBS, red stars DF12 10 percent FBS, green stars with DMEM 5 percent FBS and purple stars with DF12 5 percent FBS cell culture condition

Table 4.9. Tentative SERS Band Assignments [353,387,388]

cm^{-1}	Assignment
501	$\nu(\text{S-S})$ gauche-gauche-gauche
524	S-S disulfide stretching in proteins
548	Cholesterol
573	Cytosine, guanine
596	Phosphatidylinositol
618	C-C twisting (protein)
623	C-C twist aromatic ring
636	C-S stretching & C-C twisting (Proteins)
653	C-C twisting (Tyrosine)
662	C-S stretching mode of cystine (collagen type I)
666	G, T (ring breathing modes in the DNA bases)
678	Ring breathing (DNA bases)

708	v(C-S) trans
755	Symmetric breathing (Tryptophan)
800	Phosphate ion interactions
813	C5'-O-P-O-C3' phosphodiester bands in RNA
838	Ring breathing (Tyrosine)
882	Protein
898	Adenine
904	C-C skeletal stretching
906	Tyr
914	Ribose
951	vs (CH ₃) of proteins (α -helix)
972	Lipid
1002	Phenylalanine
1012	Ring breathing (Tryptophan)
1030	Phenylalanine, C-N stretching (Proteins)
1060	Chain C-C stretching (lipids)
1130	Phospholipid structural changes (trans versus gauche isomerism)
1155	C-C (and C-N) stretching (Proteins)
1180	Tyrosine, Phenylalanine
1199	Ring breathing (Tryptophan)
1218	Protein
1235	Amide III
1272	Protein (α -helix)
1281	Amide III (α -helix)
1317	Amide III (α -helix)
1355	Protein (β -sheet)
1391	C-H rocking
1418	C-H deformation
1444	Cholesterol
1558	Trp
1600	C=O stretching (Amide I)

4.2.5. Statistical Analysis

For the observation of the variation of the SERS spectra, PCA and LDA was applied to the obtained SERS spectra of SC-AuNPs, HC-AuNPs complex and intracellular SERS spectra of four cell lines as seen in Figure 4.16., 4.17. and 4.18. When SERS spectra of SC-AuNPs statistically investigated by LDA, it was found that t=0 was separated from all other time points in each cell culture conditions which was also reflected as distinct spectral pattern in SERS spectra. On the other hand, with increasing time better separation between the time points was achieved and 16 and 24 h interaction time of AuNPs with cell culture media provided higher signal intensity thus better separation from other time points. When

different cell culture conditions were compared, better separation achieved by DMEM with 5 percent FBS where each time point provided different location in the LDA spots. These results show that SERS can significantly distinguish PC formation on the AuNPs for different cell culture conditions.

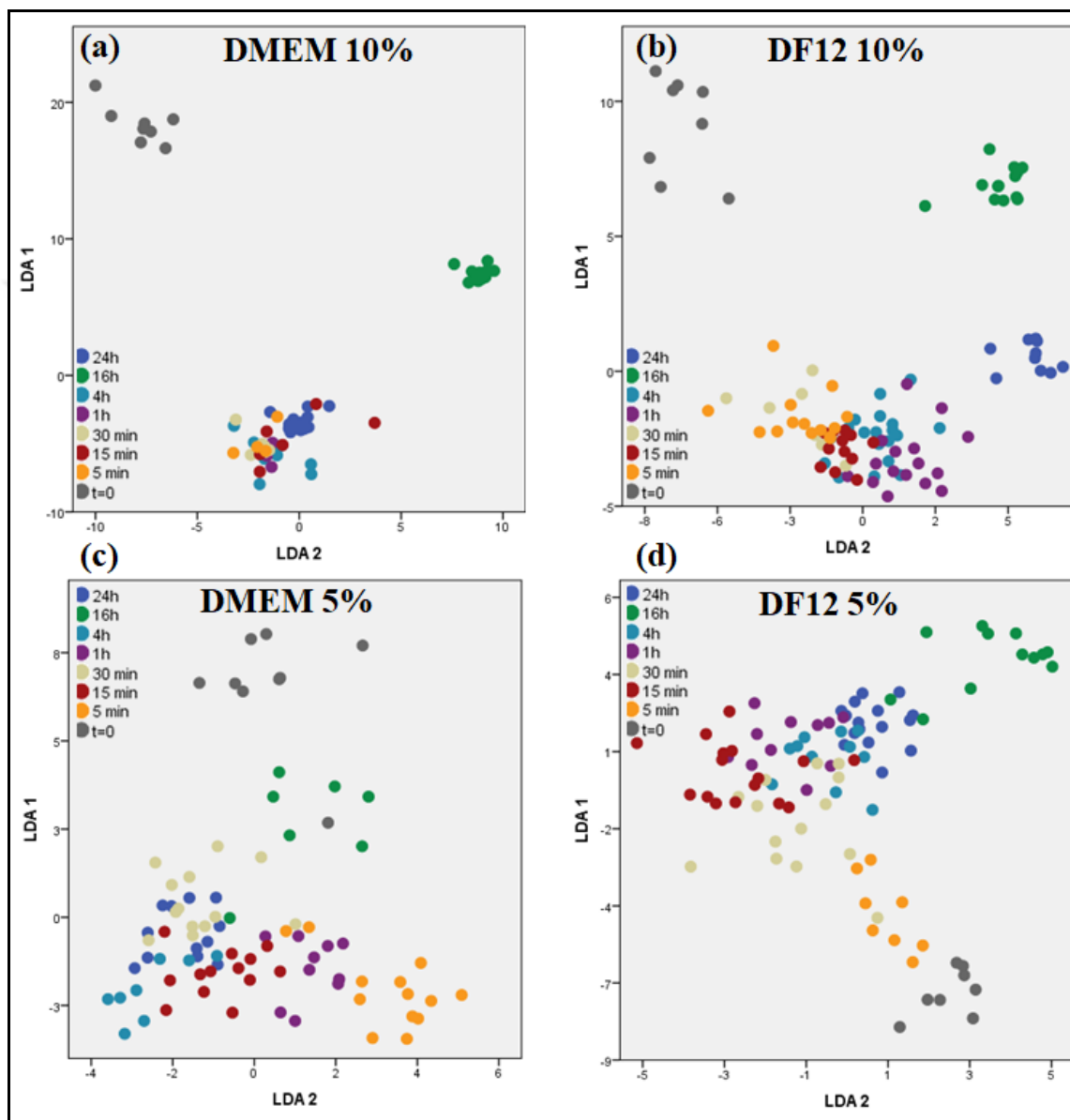


Figure 4.16. LDA Plots of Protein Corona on AuNPs without any centrifugation (SC) in (a) DMEM with 10 percent FBS, (b) DMEM:F12 (DF12) with 10 percent FBS, (c) DMEM with 5 percent FBS and (d) (b) DMEM:F12 with 10 percent FBS

Similar with the SC measurements, variation of the SERS spectra obtained from HC-AuNPs complex was examined by LDA as seen in Figure 4.17. When compared with the LDA plots of SC-AuNPs complex, better separation was achieved due to the elimination of surrounding environment from the AuNPs surface and better signal achievement with SERS. SERS spectra belong to the each time point separated better which means obtained SERS spectra has ability to distinguish time dependent changes in the HC structure. Moreover, when cell culture conditions were compared DMEM with 5 percent FBS provided separated groups of time points similar with SC measurements.

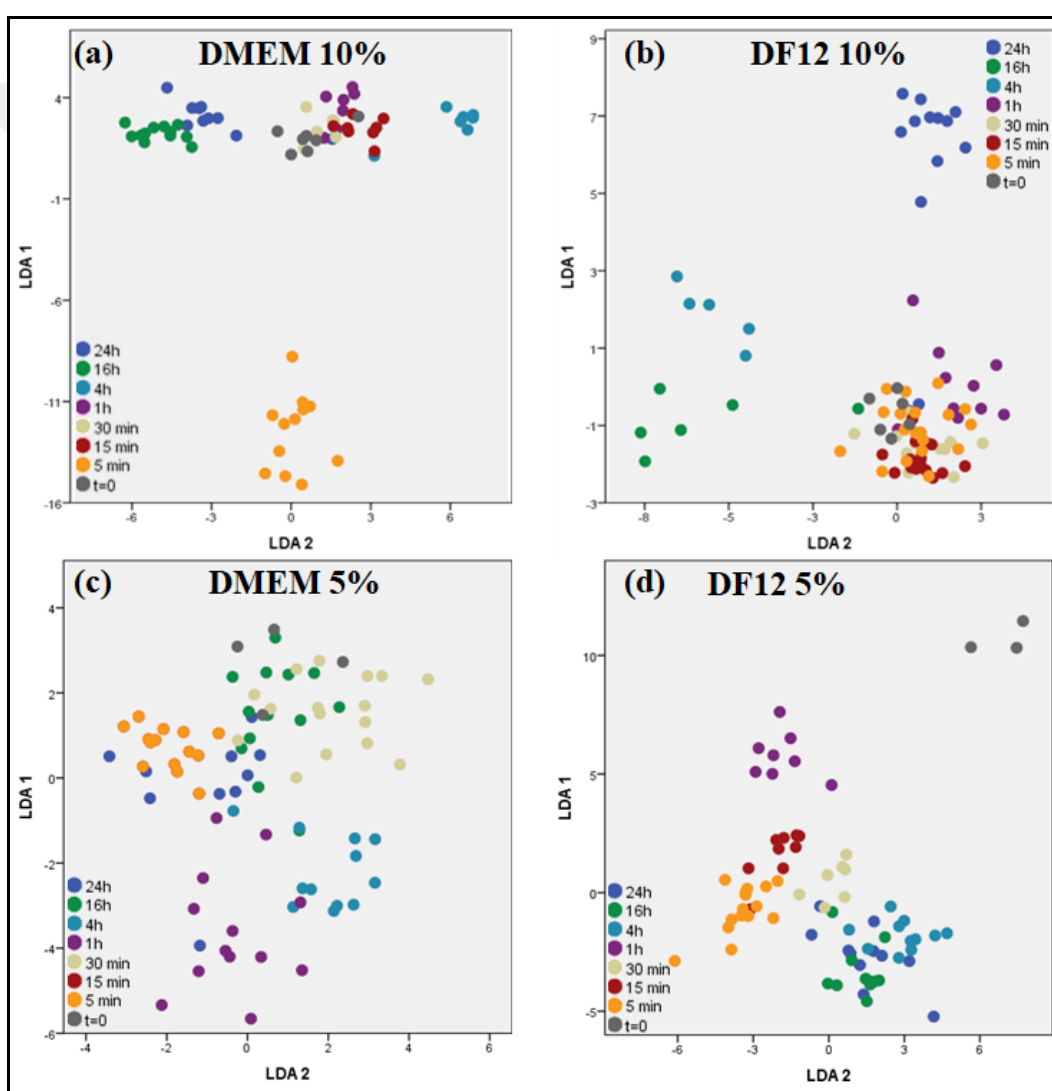


Figure 4.17. LDA Plots of Protein Corona on AuNPs after centrifugation (HC) in (a) DMEM with 10 percent FBS, (b) DMEM:F12 (DF12) with 10 percent FBS, (c) DMEM with 5 percent FBS and (d) (b) DMEM:F12 with 10 percent FBS

After the statistical analysis of PC formation on AuNPs, intracellular SERS spectra based on different cell culture conditions and PC was also evaluated statistically by LDA as seen in Figure 4.18. In each cell line, using different cell culture conditions provided separation in the LDA plots which shows the ability of the SERS to distinguish PC dependent spectral changes on each cell type.

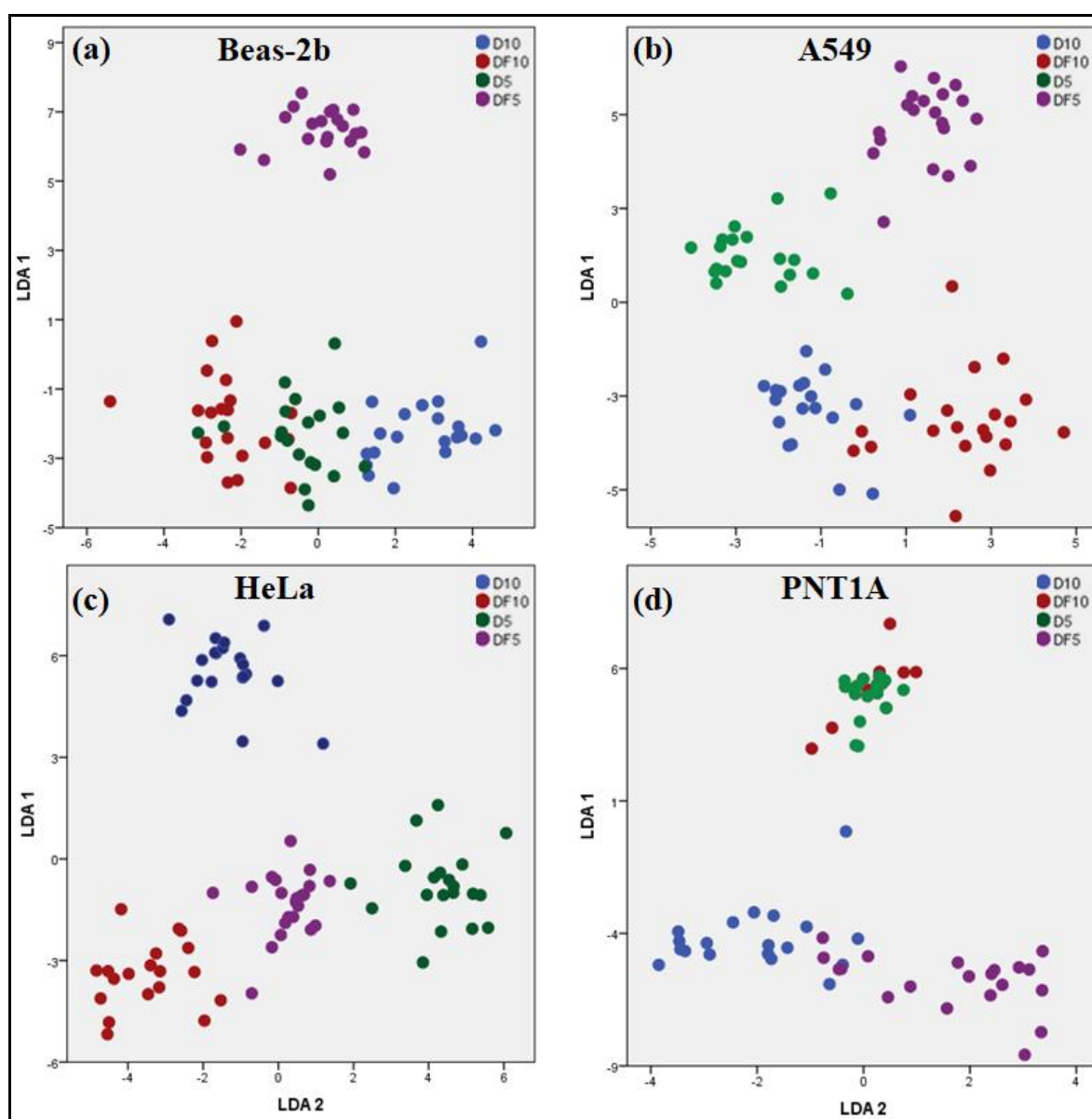


Figure 4.18. LDA Plots of (a) Beas-2b, (b) A549, (c) HeLa, and (d) PNT1A cells treated AuNPs in DMEM with 10 percent FBS (D10), DMEM:F12 with 10 percent FBS (DF10), DMEM with 5 percent FBS (D5), DMEM:F12 with 5 percent FBS (DF5)

4.3. ENDOCYTOSIS INHIBITION STUDIES

4.3.1. Choice of Inhibitors

To internalize macromolecules and particles, cells utilize phagocytosis (cell eating) and pinocytosis (cell drinking) including macropinocytosis, clathrin, caveolae, Arf6, flotilin, Cdc42 and RhoA dependent endocytosis [15,35,99,101]. These pathways operate using different mechanisms. For instance, clathrin dependent endocytosis is ended with the lysosomal decomposition while caveolae dependent endocytosis is ended with non-lysosomal (subcellular) compartments [178].

From the AuNPs uptake studies, it is known that uptake of AuNPs is dependent on the size, shape and surface chemistry of the NPs [206-208]. 50 nm AuNPs are mainly internalized by the clathrin dependent endocytosis but it is not the only pathway used for the uptake due the relatively size range of synthesized AuNPs [209,210].

For the investigation of the AuNPs endocytosis pathway, 9 different inhibitors were used for the inhibition of different pathways as shown in Table 4.10. Moreover, for one pathway more than one inhibitor were used due to their non-selective inhibitory effects on intracellular processes and other endocytic mechanisms [389].

Table 4.10. Inhibitors used for the endocytosis of nanomaterials

Agent	Inhibited Endocytosis Pathway	Effect	REF
NaN₃	Receptor Mediated	ATP depletion	[209]
Deoxyglucose	Receptor Mediated	ATP depletion	[363]
Amiloride	Macropinocytosis	Lowering submembranous pH	[34, 390, 391]
Cytochalasin D	Macropinocytosis	Disruption of F-actin (not specific)	[34, 171, 211]

Sucrose	Clathrin dependent	Interaction of clathrin and adaptors by inhibiting the formation of shallow-coated pits (type I-coated pits) or receptor clustering	[209, 211]
Chlorpromazine	Clathrin dependent	inhibiting the progression from type II-coated pits to type III-coated pits (specific)	[34, 363, 392]
Nystatin	Caveolae dependent	Perturbation of cholesterol function	[25, 34, 393]
Filipin	Caveolae dependent	Perturbation of cholesterol function	[25, 100, 392]
Dynasore	Dynamin dependent	Blocking vesicle scission by the inhibition of dynamin	[25, 99, 211]

4.3.1.1. Sucrose (Scr)

Hypertonic (0.4 - 0.5 M) sucrose (Scr) inhibits the dispersion of clathrin lattices on the plasma membrane [394]. Although Scr is a commonly used inhibitor for the clathrin mediated endocytosis (CIME), it also affects other endocytosis pathways and cellular mechanisms. Carperntier et al. showed that it is not only reducing clathrin coated pits on the membrane but also lead the removal of non-coated invaginations and also blockage of macropinocytosis [395]. As cellular mechanisms, it can cause cell shrinkage which is ended up with the changed activity of plasma membrane ion transporters, pumps and channels [396]. Moreover, induction of ion transporters activates kinases that may lead to the changing of the cortical actin cytoskeleton.

SERS spectra of Scr along with A549 cells and Scr treated A549 cells SERS spectra with the structure of Scr are shown in Figure 4.19. As seen, Scr does not interfere with the SERS spectrum from cells.

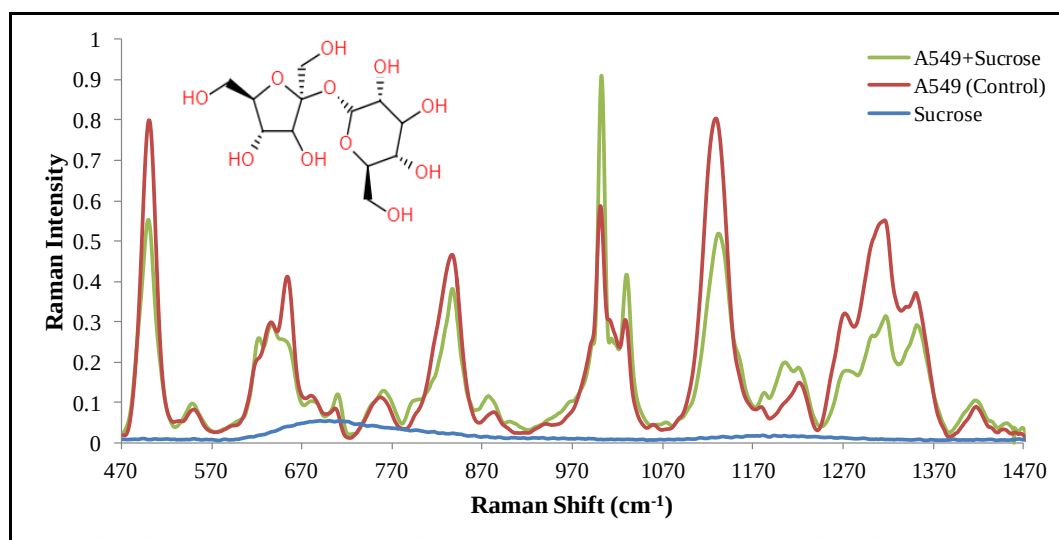


Figure 4.19. Chemical structure of Scr and SERS spectrum of Scr (500 mM), control A549 cells and Scr treated A549 cells

4.3.1.2. Chlorpromazine (Cpm)

Chlorpromazine (Cpm) is a cationic amphipathic drug, which is used in the concentration range of 50-100 μM to disable clathrin and AP2 adaptor complex on the cellular membrane. It is more specific inhibitor than Scr thanks to not affecting lipid raft/caveolae mediated endocytosis (CaME). However, it can still interact with lipid bilayers and increase lipid fluidity within the plasma membrane related to the interference of biogenesis of large intracellular vesicles such as phagosomes and macropinosomes [397].

Chemical structure of Cpm and SERS spectrum of only Cpm, A549 cells and Cpm treated A549 cells are shown in Figure 4.20. As seen, obtained spectra from Cpm interfere with the SERS spectrum obtained from cells at 536 cm^{-1} , 556 cm^{-1} , 587 cm^{-1} , 678 cm^{-1} , 770 cm^{-1} , 1167 cm^{-1} and 1250 cm^{-1} . The spectral interpretations of the peaks for the endosomal choice of AuNPs were made cautiously at these regions.

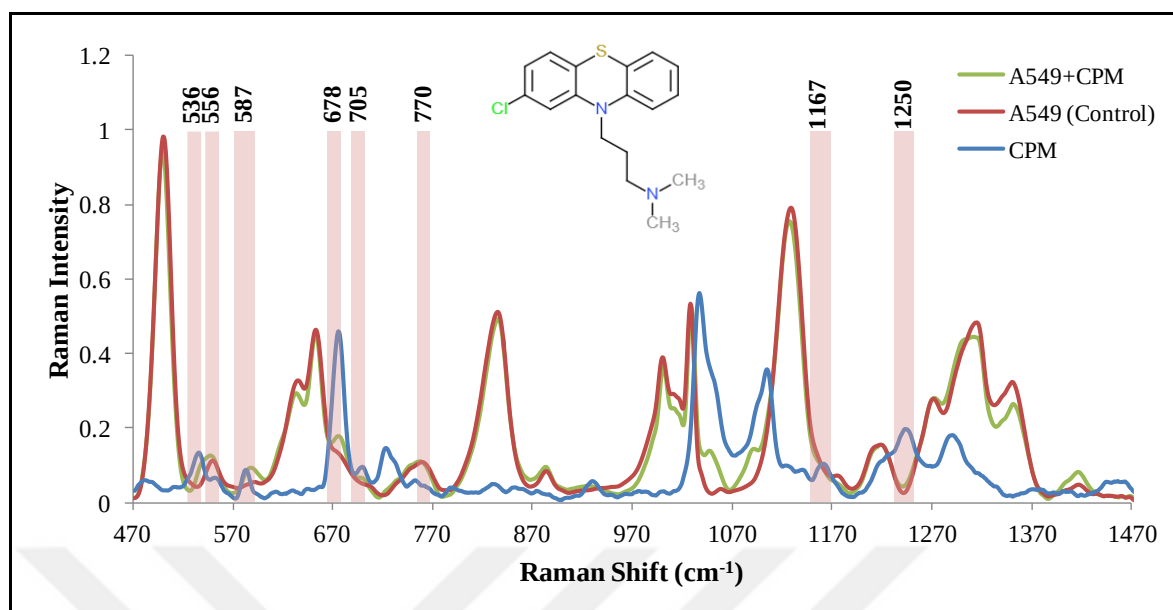


Figure 4.20. Chemical structure of Cpm and SERS spectrum of Cpm (100 mM), control A549 cells and Cpm treated A549 cells

4.3.1.3. Nystatin (Nys)

Nystatin (Nys) is a polyene antibiotic that affects cholesterol-rich domains. It provides formation of large aggregates in the membrane which accumulate and separate cholesterol from membrane. With that effect, cholesterol rich domains of the plasma membrane deform and this causes changes in caveolae and GPI-anchored proteins which is ended up the inhibition of lipid raft ligands internalization [389,398-400].

Chemical structure of Nys and SERS spectrum of only Nys, A549 cells and Nys treated A549 cells are shown in Figure 4.21. As seen, Nys does not interfere with the SERS spectrum from cells.

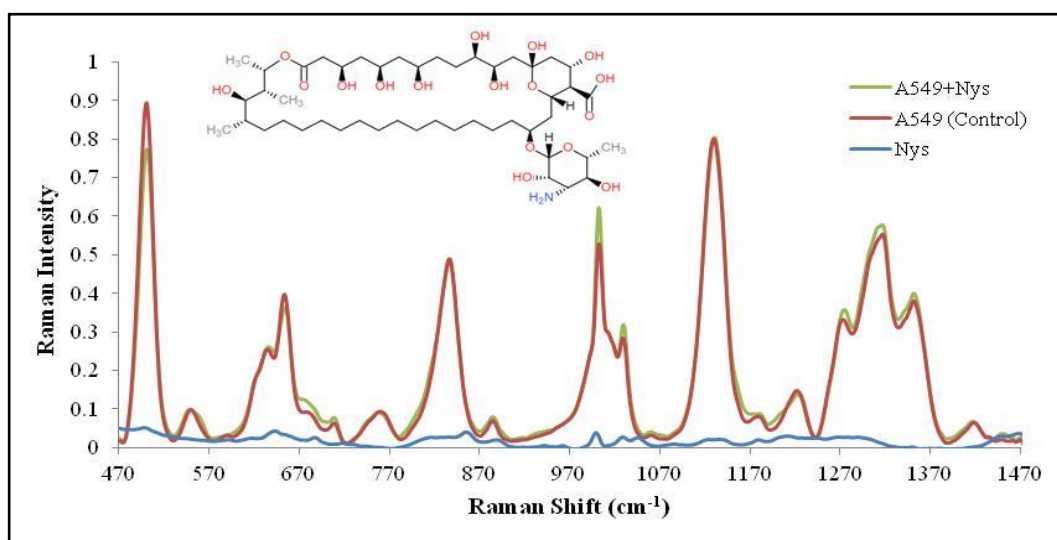


Figure 4.21. Chemical structure of Nys and SERS spectrum of Nys (0.4 μM), control A549 cells and Nys treated A549 cells

4.3.1.4. *Filipin (Fil)*

Fil is a polyene antibiotic as such Nys which affects cholesterol-rich domains. Lipid rafts are disrupted by Fil with the formation of large aggregates that cholesterol accumulated. With the separation of cholesterol, cholesterol rich domains of plasma membrane are disrupted which is resulted as the blockage of endocytosis from lipid rafts. Working principle of Fil and Nys are similar but Fil was found as more effected with changing cell permeability due to interaction with membrane phospholipids [401] and disruptions of the F-actin linkage and plasma membrane [402]. Moreover, with the changed structure of membrane, Fil also affects caveolae and GPI-anchored proteins [389,398-400].

Chemical structure of Fil and SERS spectrum of only Fil, A549 cells and Fil treated A549 cells are shown in Figure 4.22. As shown, Fil does not interfere with the SERS spectrum from cells.

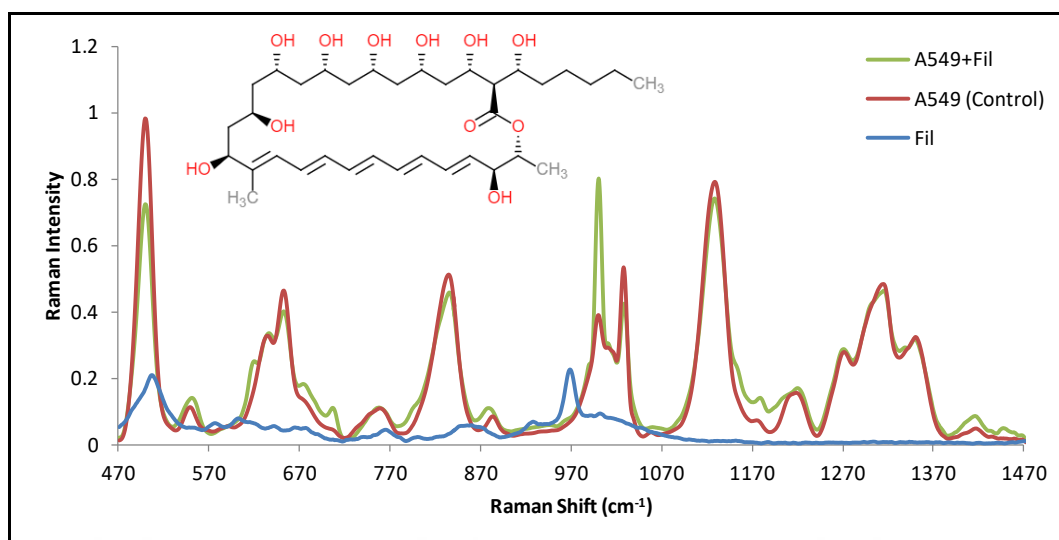


Figure 4.22. Chemical structure of Fil and SERS spectrum of Fil (2 μ M), control A549 cells and Fil treated A549 cells

4.3.1.5. *Dynasore (Dyn)*

Dynasore is an inhibitor which blocks vesicle scission by the inhibition of dynamin protein. With the inhibition of dynamin protein, both clathrin and caveolae mediated endocytosis can be eliminated, because dynamin protein has role in both endocytosis pathways to help vesicle scission and formation of early endosomes. Furthermore, among the used inhibitors, Dyn is the most specific agent that is used for the inhibition of endocytosis pathways [403].

Chemical structure of Dyn and SERS spectrum of only Dyn, A549 cells and Dyn treated A549 cells are shown in Figure 4.23. As seen, Dyn does not interfere with the SERS spectrum from cells.

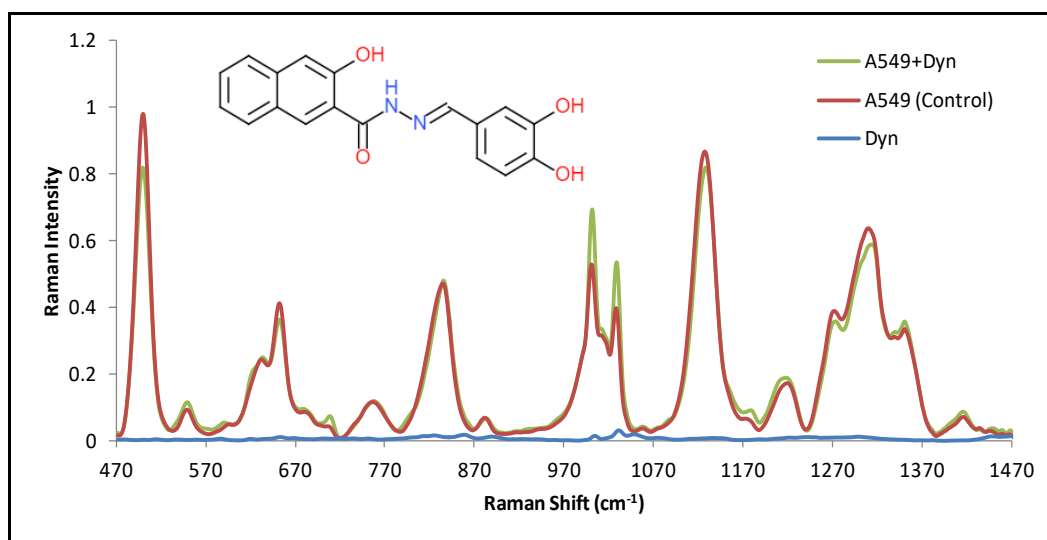


Figure 4.23. Chemical structure of Dyn and SERS spectrum of Dyn (80 nM), control A549 cells and Dyn treated A549 cells

4.3.1.6. Sodium Azide (NaN_3)

NaN_3 is used for the ATP depletion which is achieved by mitochondrial oxidative phosphorylation [404]. It inhibits not only cytochrome c oxidase but also ATP hydrolase activity by inhibiting ATPases such as ABC transporters [405,406], the preprotein translocase SecA [407,408], DNA topoisomerase II α [409], and ecto-ATPases [410]. On the other hand, it affects potassium channels and induce ROS production and apoptosis with effecting peroxisome proliferator-activated receptor γ co-activator 1- α (Pgc-1 α)-associated signaling pathways [411-416].

Chemical structure of NaN_3 and SERS spectrum of only NaN_3 , A549 cells and NaN_3 treated A549 cells are shown in Figure 4.24. As shown, obtained spectra from NaN_3 interfere with the SERS spectrum from cells in some peaks such as 1004 cm^{-1} , 1035 cm^{-1} and 1231 cm^{-1} . The spectral interpretations of the peaks for the endosomal choice of AuNPs were made cautiously at these regions.

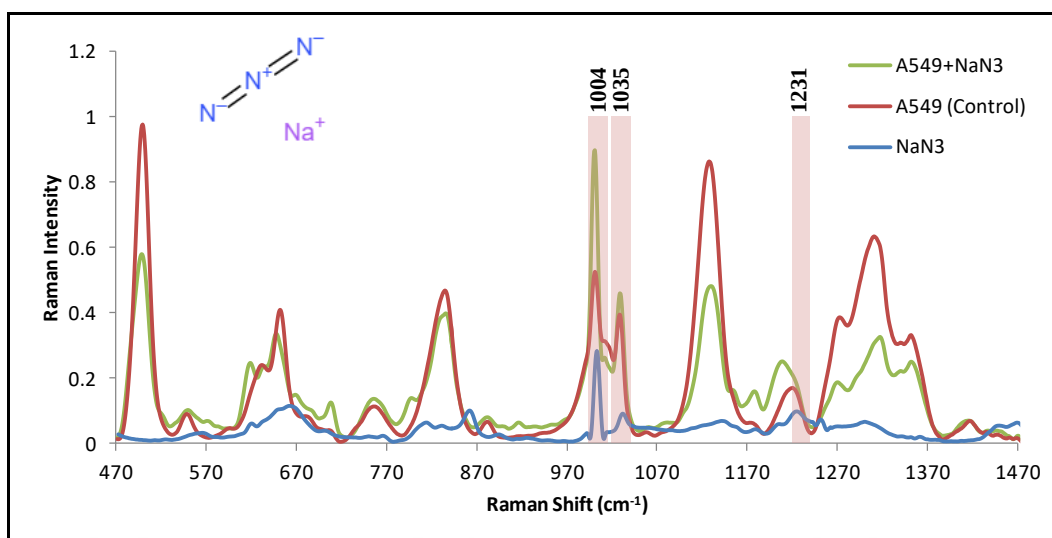


Figure 4.24. Chemical structure of NaN_3 and SERS spectrum of NaN_3 (100 mM), control A549 cells and NaN_3 treated A549 cells

4.3.1.7. 2-Deoxy-D-glucose (dG)

2-deoxy-D-glucose (dG) is a synthetic glucose analog where hydroxyl group on the second carbon is replaced by hydrogen. It is a well-known ATP depletion and chemotherapeutic agent which not only depletes energy but also inhibits glycolysis, induces mitochondrial reactive oxygen species (mROS), interferes with N-linked glycosylation and induces autophagy as dose dependent manner [417-419].

Chemical structure of dG and SERS spectrum of only dG, A549 cells and dG treated A549 cells are shown in Figure 4.25. As seen, dG does not interfere with the SERS spectrum from cells.

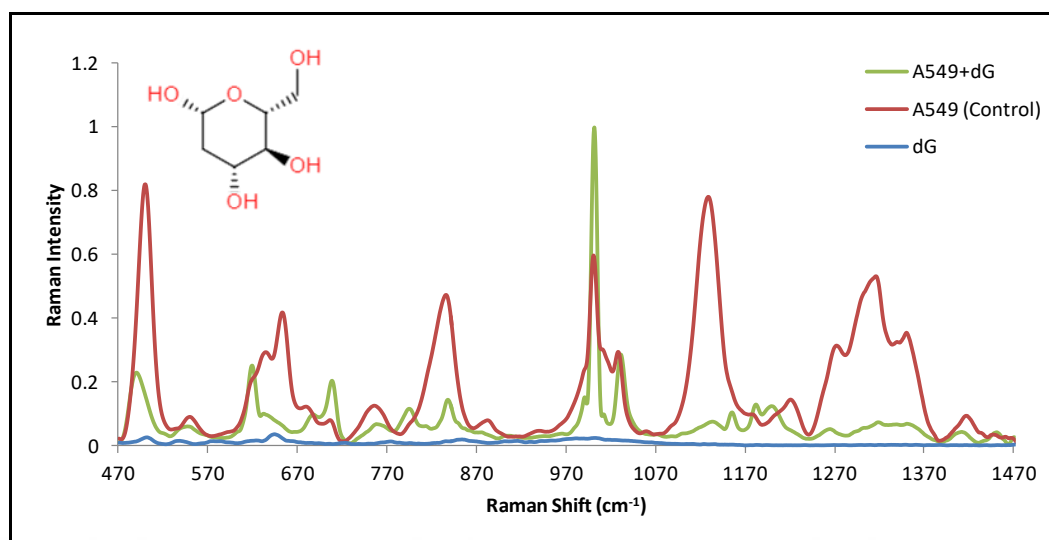


Figure 4.25. Chemical structure of dG and SERS spectrum of dG (100 mM), control A549 cells and dG treated A549 cells

4.3.1.8. Cytochalasin D (CytoD)

CytoD is used for the inhibition of macropinocytosis by blocking actin polymerization via occupying a fast-growing (barbed) ends of actin filaments and also interacting with capping proteins (CAPZA1 and capping protein α subunit family) [420]. Actin polymerization is blocked by CytoD in two major steps. In nucleation step, it binds to the barbed ends of actin filaments which oligomerization reaction occur, and elongation step cannot occur while barbed ends are occupied by CytoD [421]. Furthermore, CytoD can also affect the ClME due to the role of actin in also ClME [422]. In CaME, actin does not play any role; however, recycling of the endosomes back to the plasma membrane is also regulated by actin [171].

Chemical structure of CytoD and SERS spectrum of only CytoD, A549 cells and CytoD treated A549 cells are shown in Figure 4.26. CytoD SERS spectra interfere with cellular SERS spectrum at 1150 cm^{-1} and 1271 cm^{-1} but there was not any increase caused by CytoD interference at CytoD treated cellular SERS spectrum. Please note that CytoD was obtained from a dried droplet area, where the spectral bands could be more intense compared to the possible interfering bands when CytoD molecules are in cells.

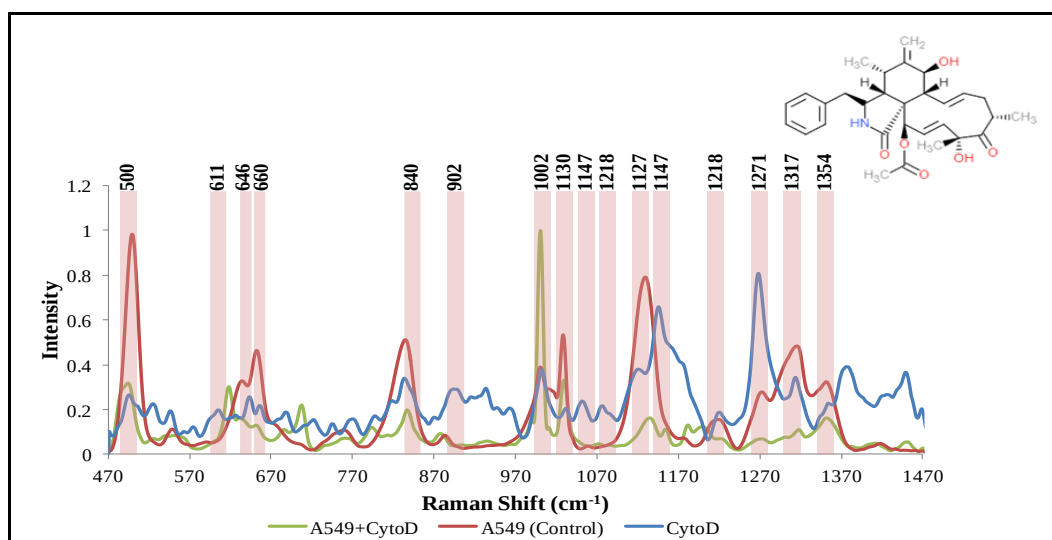


Figure 4.26. Chemical structure of CytoD and SERS spectrum of CytoD (10 μ M), control A549 cells and CytoD treated A549 cells

4.3.1.9. Amiloride (Aml)

Aml, a guanidinium containing pyrazine derivative, is an inhibitor of Na^+/H^+ exchangers. It blocks macropinocytosis by inhibiting of Na^+/H^+ exchanger which hinders the elimination of intracellular excess H^+ . As a result, intracellular acidification becomes impaired [423, 424]. This impaired acidification affects signaling and cytoskeleton arrangements and macropinocytosis. On the other hand, Aml caused acidification inhibits the activation of Rac1/CDC42 and their effectors which are play role in actin polymerization via the activation of actin nucleation promoting complexes such as the SCAR/WAVE complex and WASP [425,426]. Impaired acidification could also affects CLME when pH is lower than 6.0 [427,428].

Chemical structure of Aml and SERS spectrum of only Aml, A549 cells and Aml treated A549 are shown in Figure 4.27. As shown, obtained spectra from Aml interfere with the SERS spectrum obtained from cells. The spectral interpretation of the peaks for the endosomal choice of AuNPs was made cautiously at these regions.

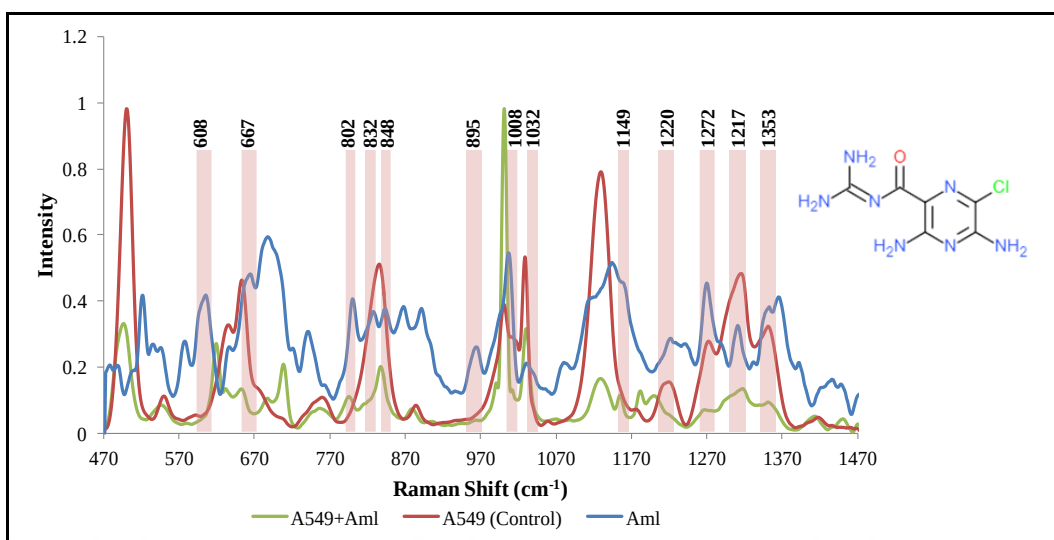


Figure 4.27. Chemical structure of Aml and SERS spectrum of Aml (100 μM), control A549 cells and Aml treated A549 cells

4.3.2. Optimization of Inhibition Conditions

Inhibition studies can be performed continuously and non-continuously in the presence and absence of the fetal bovine serum (FBS). To choose the optimum method, cytotoxic effect and the inhibition rate of inhibitors should be considered simultaneously.

Inhibitory effects of different inhibitors were determined using flow cytometry. Forward scatter (FSC) intensity and side scatter (SSC) intensity are used for the determination of AuNP uptake. FSC provides information about the overall size of cells while SSC provides information about internal structures and organelles. SSC is determined by the internal structure of the cell and cellular mass and SSC signal is affected by the refractive index of the cytoplasm and the number and type of organelles present in the cell. Thus, SSC can be used as a parameter to detect the internalized NPs due to their ability to increase granulation in the cells, which is resulted in higher side-scattering of light [429-432]. SSC shift of the cells with AuNPs uptake can be seen in Figure 4.28. As seen, for all type of cells, internalization of AuNPs caused higher SSC with different increase rates. To find the AuNP uptake inhibition rate, SSC shift of only AuNP treated cells was normalized as 100 percent uptake and inhibition rate of inhibitors was calculated from the comparison.

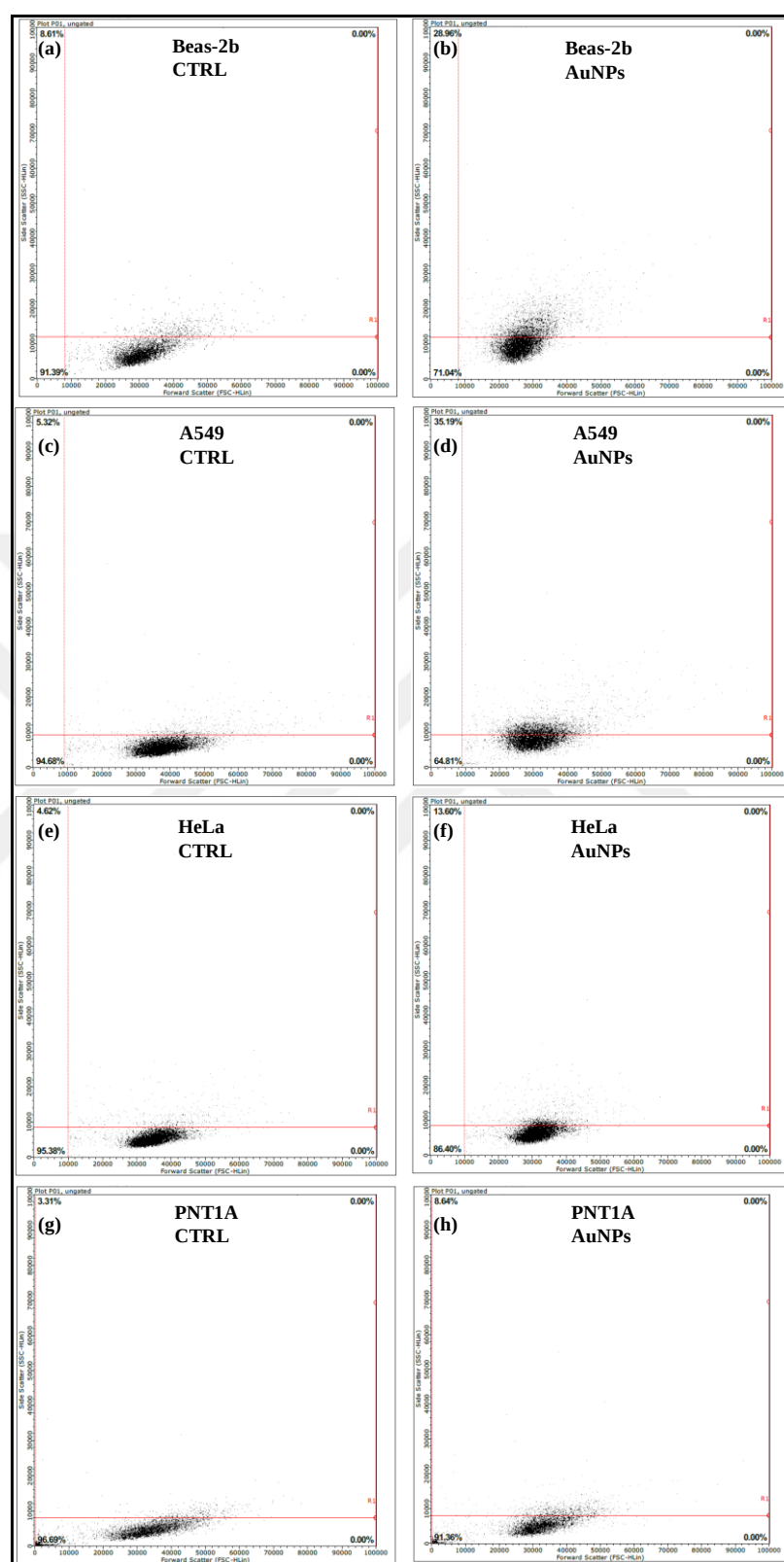


Figure 4.28. SSC Shift results of (a) Beas-2b, (b) Beas-2b treated with 24 h AuNPs, (c) A549, (d) A549 treated with 24 h AuNPs, (e) HeLa, (f) HeLa treated with 24 h AuNPs, (g) PNT1A, (h) PNT1A treated with 24 h AuNPs

Figure 4.29.a and Figure 4.29.b shows the continuous inhibition rate of the inhibitors in the presence of FBS for A549 and Beas-2b cell lines, respectively. It can be seen that all of the used inhibitors cause concentration dependent decrease in the internalization of the AuNPs according to the SSC shift rates. However, most of the inhibitors showed higher than 100 percent inhibition. To explain this, SSC shift of the only inhibitors was examined and the results are shown in Figure 4.29.c. It was found that inhibitors could also cause SSC shift by themselves. After this finding, all flow cytometry results are represented as normalized with excluded SSC shifts resulting from the inhibitors.

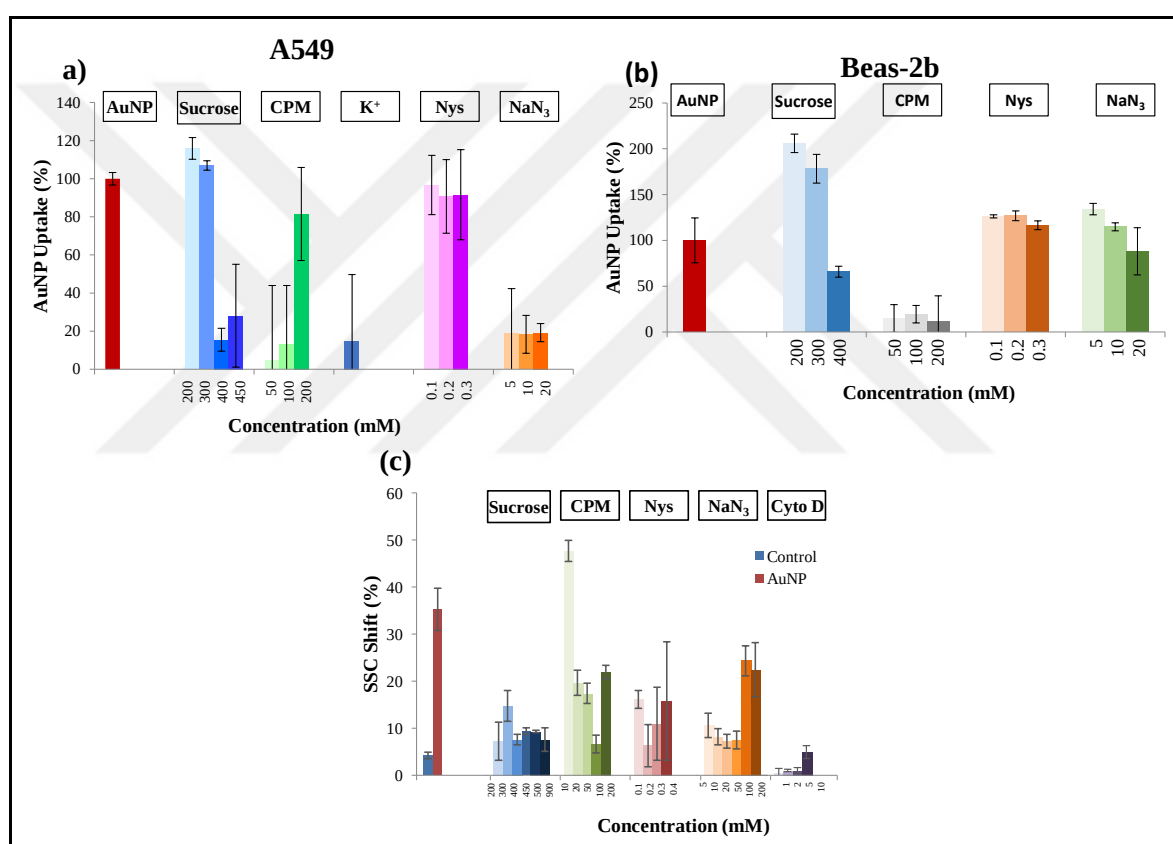


Figure 4.29. Flow cytometry results of (a) A549, (b) Beas-2b and (c) SSC shift comes from only inhibitors for A549 cells for the continuous inhibition in the presence of FBS

Normalized inhibition rates of the used inhibitors are shown in Figure 4.30. in the presence and absence of FBS. All inhibitors provide inhibition of the AuNP internalization, which means AuNPs not only uptaken by CIME but also CaME and macropinocytosis. Moreover, the effect of FBS was found as important parameter for the inhibition. Although it seems to

be FBS free treatment of inhibitors is more effective as seen Figure 4.30.b, it should be considered with cytotoxicity results of inhibitors.

Furthermore, it should be noted that, although normalized SSC shift results were used with the elimination of granulation coming from inhibitors for the investigation of AuNPs uptake rate, again higher SSC shift rates were also observed when compared with control groups. This situation was probably caused by the differential effect of the inhibitors in the presence and absence of AuNPs. Even only inhibitor treatments were used for the subtracting the over SSC shift compared with control, it was not enough for some cases to represent the SSC shift coming from only inhibitor when AuNPs were also internalized.

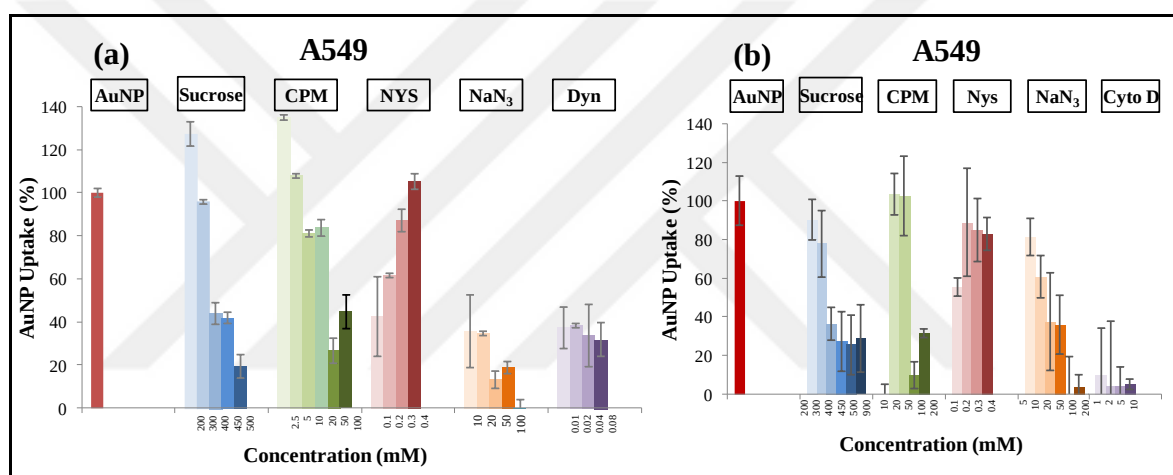


Figure 4.30. Flow cytometry results for the AuNP uptake continuous inhibition a) in the presence of FBS b) in the absence of FBS

From the cytotoxicity results, which can be seen in Figure 4.31., it was found that continuous treatment even in the presence of FBS was not an efficient way for inhibition owing to the toxicity of inhibitors. Due to toxicity obtained for the inhibition in the presence of FBS, cytotoxicity of FBS-free treatment was not assessed which is expected as higher toxicity.

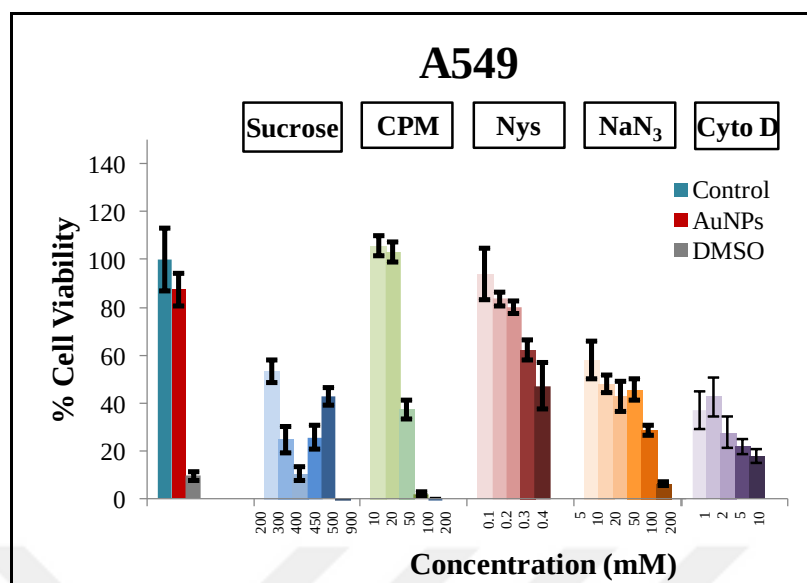
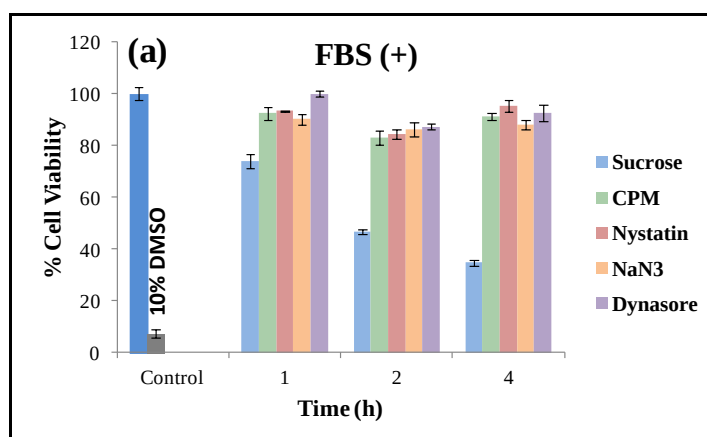


Figure 4.31. Cytotoxicity results of continuous inhibition in the presence of FBS for A549 cell line

Since the continuous inhibition is not suitable method to study internalization of AuNPs, the non-continuous method was selected as inhibition method. Different time points were selected to figure out cytotoxicity and inhibition rates. In Figure 4.32.a, cytotoxicity results showed that non-continuous inhibition in the presence of FBS is suitable. Before choosing the time point of non-continuous inhibition, flow cytometry results should be considered as seen in Figure 4.32.b and 4.32.c. From the uptake inhibition studies, both 2 and 4 h inhibition in the presence of FBS can provide inhibition rate and lower toxicity.



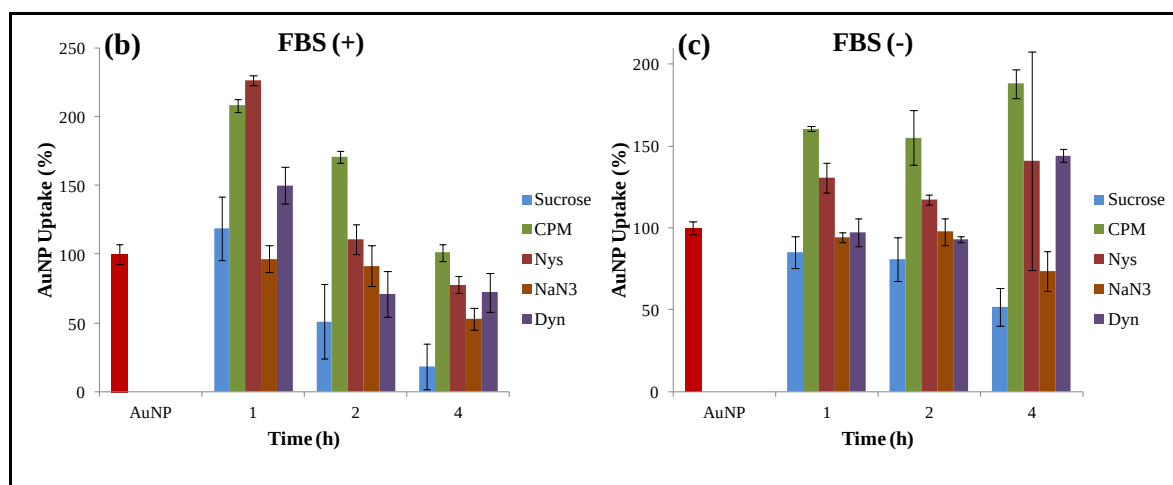


Figure 4.32. Non-Continuous inhibition for A549 cell line. (a) Cytotoxicity results of non-continuous inhibition in the presence of FBS (b) Flow cytometry results for non-continuous inhibition in the presence of FBS (FBS (+)) and (c) Flow cytometry results for non-continuous inhibition in the absence of FBS (FBS (-))

For the selection of the time point between 2 and 4 h, healthy cell line Beas-2b was also considered as seen in Figure 4.33. Figure 4.33.a and 4.33.b show the cytotoxicity results of inhibition studies for Beas-2b cell line in the presence and absence of the FBS. When uptake inhibition of AuNPs at 1 and 2 h (Figure 4.33.c) was also considered, the best option was found as 2 hours inhibition in the presence of FBS with lowest toxicity rates and higher uptake reduction rates. After the optimization studies, the method was selected as 2 h non-continuous inhibition in the presence of FBS. After this optimization, the inhibition of the endocytosis was examined according to the selected method.

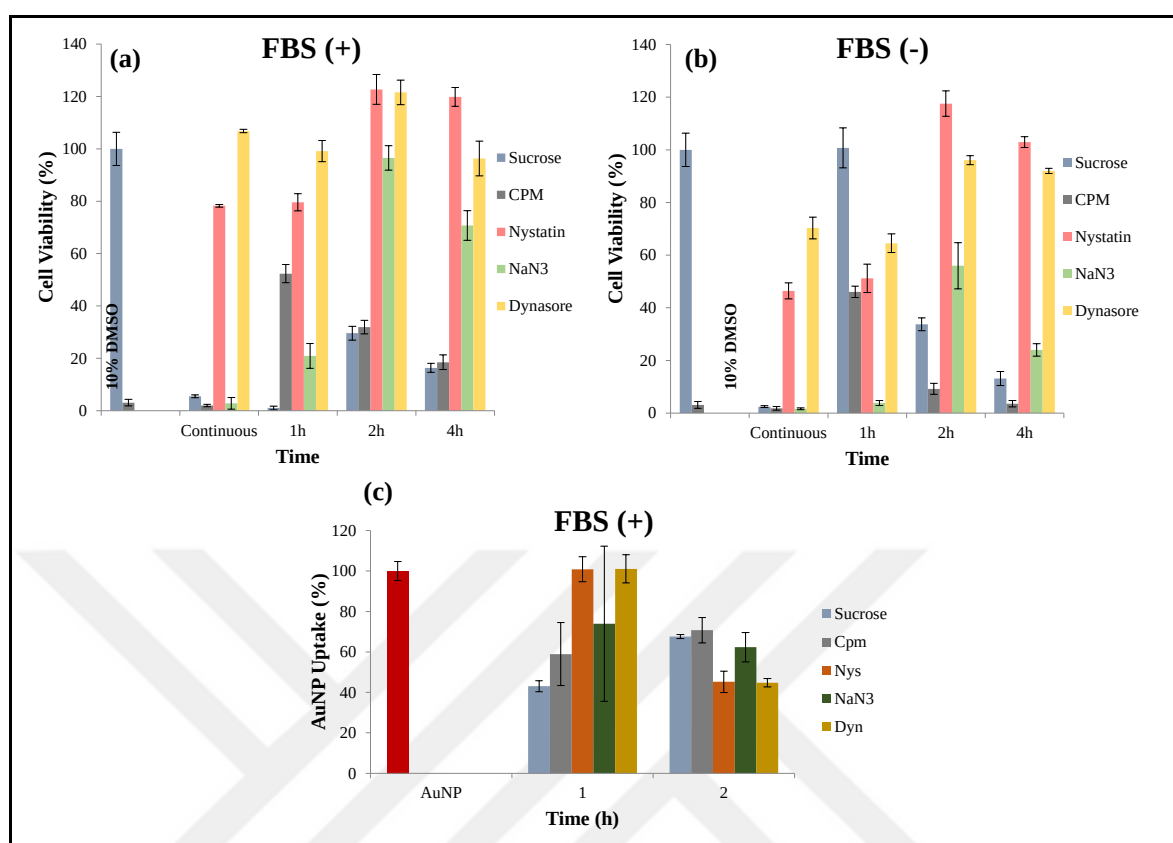


Figure 4.33. Non-Continuous inhibition for Beas-2b cell line. (a) Cytotoxicity results of non-continuous inhibition in the presence of FBS (b) Cytotoxicity results in the absence of FBS. (c) Flow cytometry results for non-continuous inhibition in the presence of FBS

4.3.3. Inhibition of Clathrin Mediated Endocytosis

Clathrin mediated endocytosis (CIME) of AuNPs were inhibited using sucrose (Scr) and chlorpromazine (Cpm). Hypertonic (0.4 - 0.5 M) Scr can inhibit the dispersion of clathrin lattices on the plasma membrane [394]. Although Scr is a commonly used inhibitor for the CIME, it also affects other endocytosis pathways and cellular mechanisms. Carpentier et al. showed that it is not only reducing clathrin coated pits on the membrane but also lead the removal of non-coated invaginations and blockage of macropinocytosis [395]. As cellular mechanisms, it can cause cell shrinkage which is ended up with the changed activity of plasma membrane ion transporters, pumps and channels [396]. Moreover, induction of ion transporters activates kinases that may lead to the changing of the cortical actin cytoskeleton.

As another inhibitor for ClME, Cpm is a cationic amphipathic drug which is used in the concentration range of 50-100 μ M to loss of clathrin and AP2 adaptor complex from the cell surface. It is more specific inhibitor than Scr thanks to not affecting lipid raft/CaME. However, it can still interact with lipid bilayers and increase lipid fluidity within the plasma membrane which is related to the interference of biogenesis of large intracellular vesicles such as phagosomes, macropinosomes [397].

4.3.3.1. Cytotoxicity of Scr and Cpm

WST-8 cell proliferation results of A549, Beas-2b, HeLa and PNT1A cells treated with Scr or Cpm were given in Figure 4.34. As seen, Scr can cause dose dependent toxicity on all cell lines. It caused cell viability reduction up to 18 percent on Beas-2b cells, 54 percent on A549 cells, 46 percent on HeLa cells and 48 percent on PNT1A cells. Cpm also caused toxicity on cell lines and provided cell viability reduction up to 1.34 percent on Beas-2b cells, 61 percent on A549 cells, 29 percent on HeLa cells and 8 percent on PNT1A cells. From the clathrin mediated endocytosis inhibition, most effected cell lines were healthy cell lines Beas-2b and PNT1A with highest cell viability reduction for both of the inhibitors and cancerous cell lines HeLa and A549 were less affected when compared with healthy cell lines.

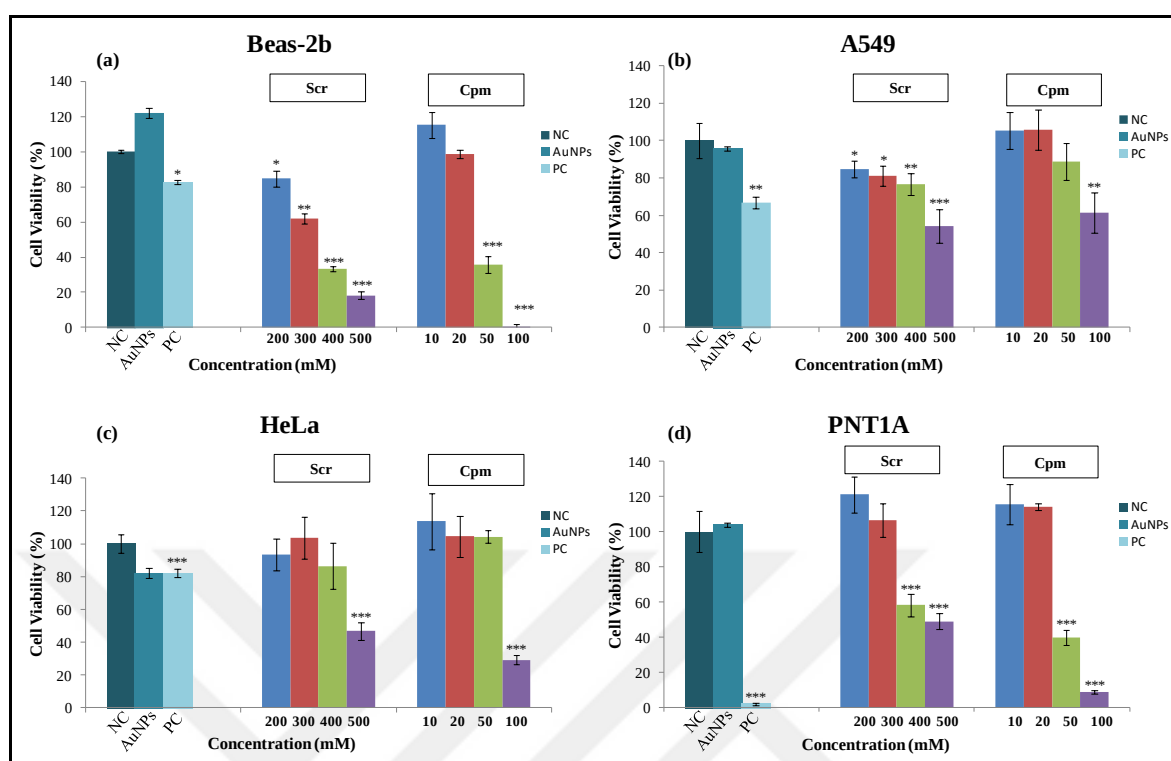


Figure 4.34. WST-8 Cell Proliferation results of (a) Beas-2b, (b) A549, (c) HeLa, and (d) PNT1A treated with Scr and Cpm. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$. PC: Positive control (10 percent DMSO was used as positive control), NC: Negative control

4.3.3.2. Uptake Inhibition by Scr and Cpm

Inhibition of the AuNPs uptake was investigated by SSC shift using flow cytometry as seen in Figure 4.35. It was found that, Scr treatment caused decrease in the SSC shift significantly from 3 cell lines up to 73, 69 and 45 percent for Beas-2b, A549 and PNT1A cells, respectively. Cpm could not provide decrease in the SSC for Beas-2b, A549, and HeLa cells and decrease in SSC shift was obtained only for the PNT1A cells up to 80 percent. These two different decrease rates of the same pathway inhibitors are caused by the nature of the used inhibitors. Cpm is a specific inhibitor that mostly affects clathrin and AP2 adaptor complex of clathrin mediated endocytosis while sucrose not only affects dispersion of clathrin lattices on the plasma membrane but also macropinocytosis, actin cytoskeleton, and plasma membrane activity. Thus, while Scr can cause SSC change for 3 cell lines due to the effect on not only endocytosis but also other mechanisms in the cell,

Cpm could not provide change in SSC except for PNT1A cell line. Consequently, for 3 cell lines AuNPs internalization rate was decreased by one of the inhibitors except for HeLa cells line, thus, it can be concluded that clathrin mediated endocytosis was one of the used pathways for the internalization of approximately 50 nm AuNPs for 3 cell types Beas-2b, A549 and PNT1A.

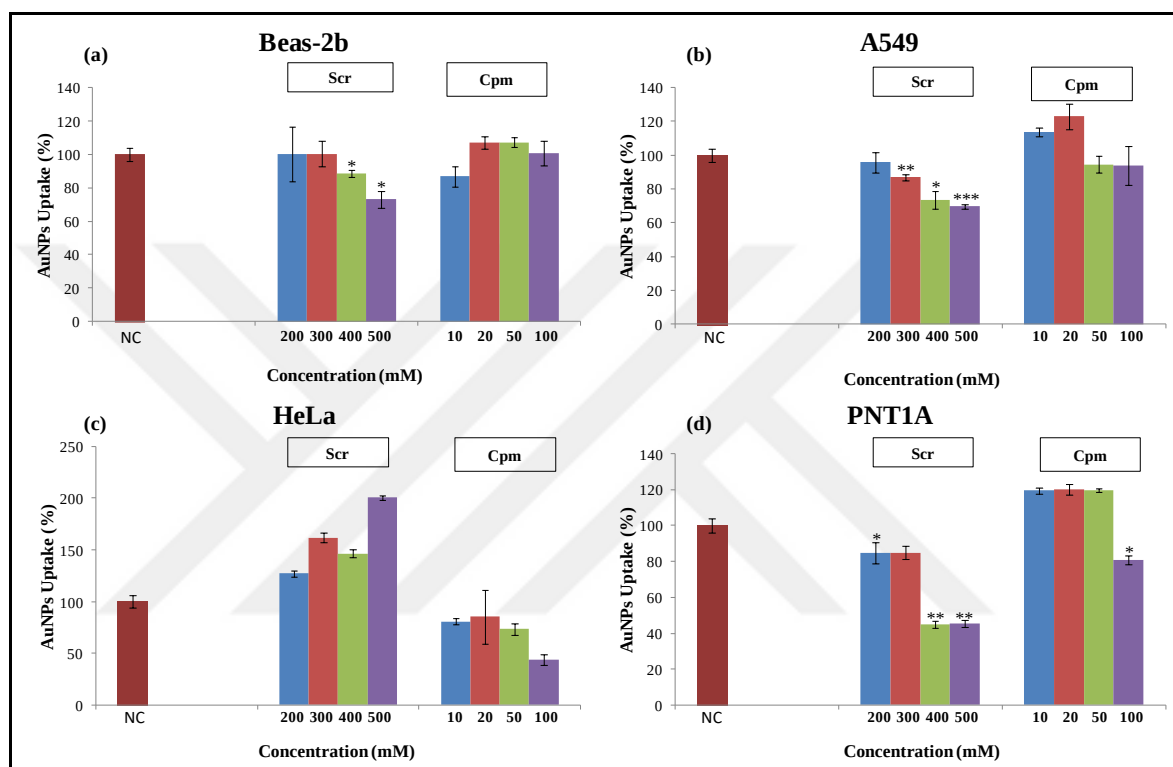


Figure 4.35. Flow cytometry analysis of a) Beas-2b, b) A549, c) HeLa, and d) PNT1A after 2 h exposure to Scr and Cpm then 22 h AuNPs. NC: Negative control

4.3.3.3. SERS Measurements

For the inhibition of ClME, Scr was used for the dispersion of clathrin lattices on the plasma [394] and Cpm was used for the loss of clathrin and AP2 adaptor complex from the cell surface [397].

Figure 4.38. shows SERS spectra of three cell lines treated with Scr. As seen, Scr treatment on three cell lines caused spectral changes. These changes could be interpreted based on the microenvironment where AuNPs reside and events where happening from a few nanometers away from AuNPs surface. AuNPs amount decrease by endocytosis inhibitory agents showed that most of the AuNPs found in different type of endosomes. With the

inhibition of endosomal pathway, obtained spectral changes should be explained by alterations on the endosomal structure. While explaining the changes in the SERS spectra, microenvironment where AuNPs found should be examined with care to see what happens in close vicinity to the AuNPs which is just a few nanometers away from AuNPs. Figure 4.36. illustrates the formation of the endosomes. As seen, there are many different biomolecules involved starting from the formation of endosomes. When the environment is examined in the AuNPs point of view; AuNPs interact with the cargo and remains inside the endosomes.

All surrounding environment changes on the endosomal membrane where AuNPs found close in a few nanometers. Thus, spectral changes could be interpreted based on the molecular changes during endosomal pathway.

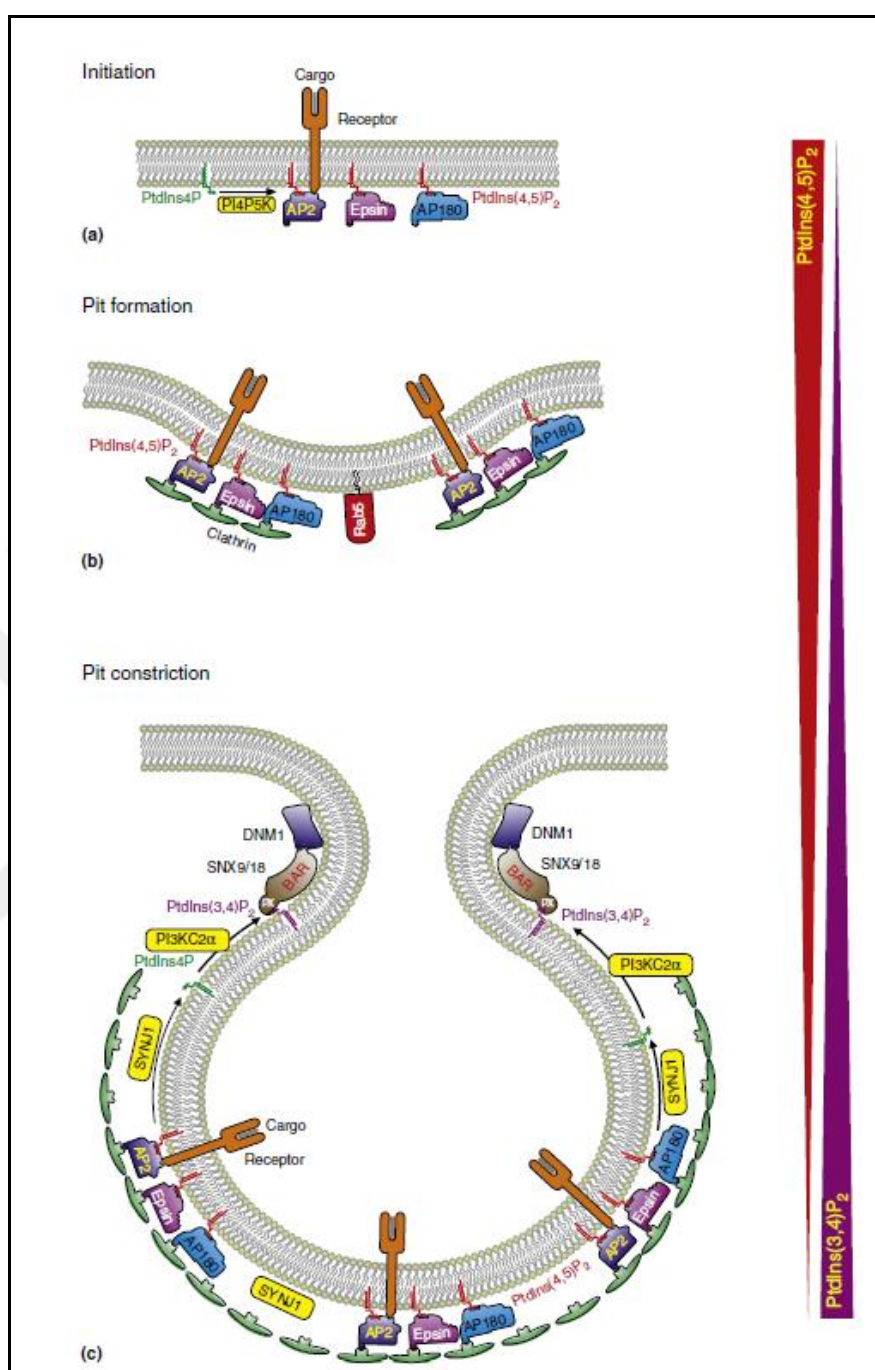


Figure 4.36. Protein profile during pit formation in the endocytosis with (a) initiation, (b) pit formation and (c) pit construction [433]

While AuNPs are inside the endosomes which in interacting with receptors, the changes occur on the endosomal membrane, obtained spectral changes could be explained by the biomolecules found on the membranes. From the starting points of endocytosis including initiation of membrane invagination, pit formation and construction; proteins and

phosphatidylinositols (PIs) are always involved in the fundamental events including, PI conversions, protein recruitment to the site and protein disassemble from the site as seen in Figure 4.37. Due to happening from a few nanometers away from AuNPs surface, changes on these events could be reflected on the obtained SERS spectra. Thus, alterations in protein recruitment, PI conversion with the use of inhibitors could be used to explain changes in the spectral bands.

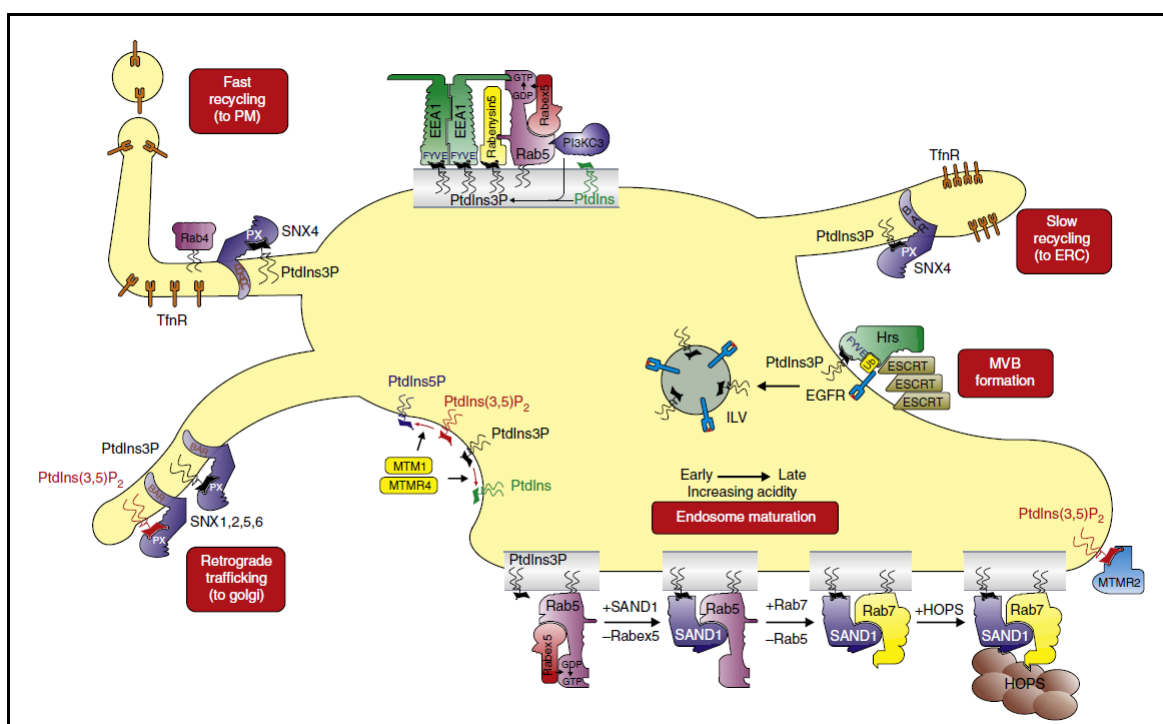


Figure 4.37. Schematic representation of endosomal maturation [433]

When SERS spectra of 3 cell lines was inspected, in all cell types, it was seen that the intensity of the peak at 1130 cm^{-1} attributed to phospholipids was found decreased significantly. Due to the elimination of the one of the internalizations pathways of AuNPs, less amount of AuNPs were internalized with only two types of endocytosis. Thus, lesser number of endosomes was formed by cells and dependently the intensity of the peak attributed to phospholipid intensity decreased.

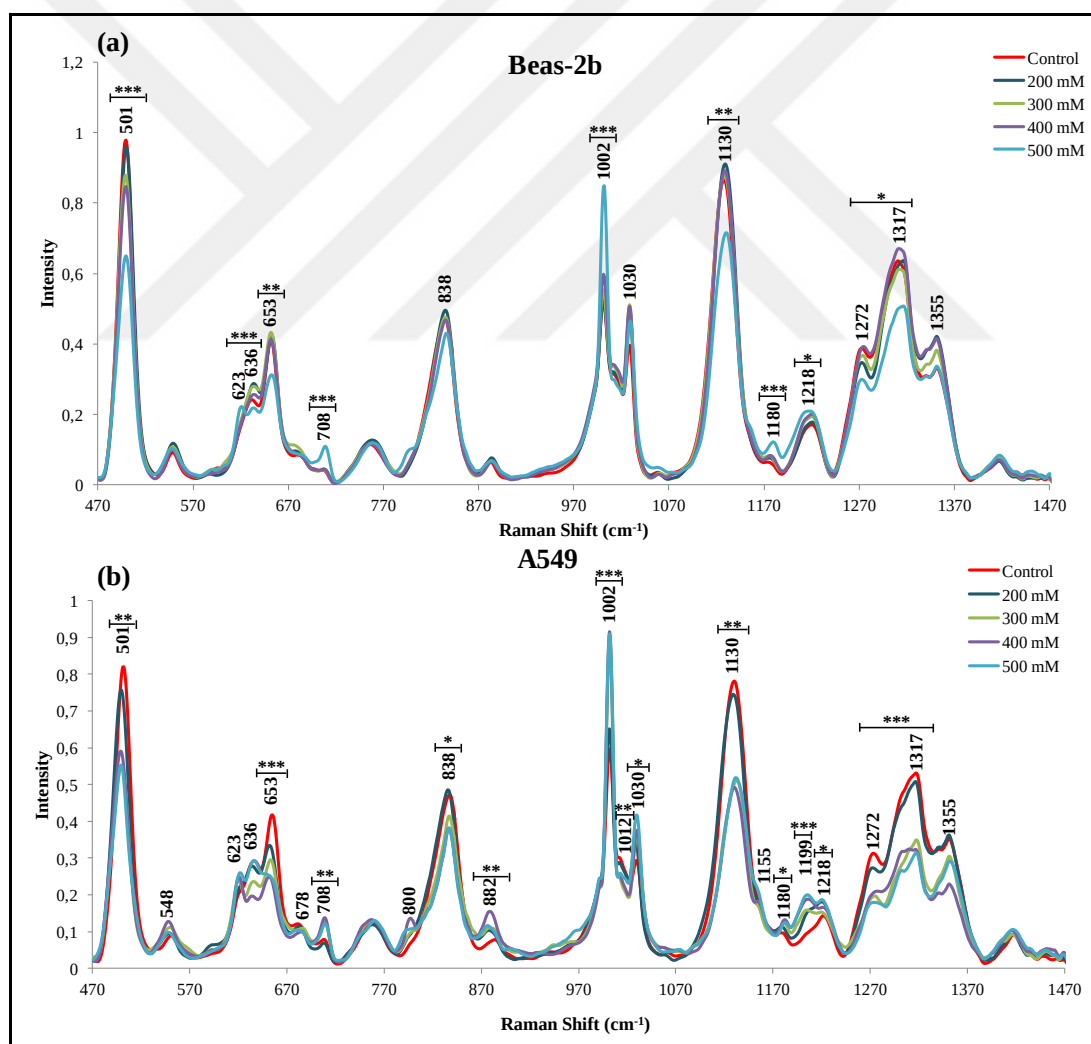
Furthermore, for all 3 cell lines, protein profile was changed with Scr treatment. In Beas-2b cells the peak intensities at 708 cm^{-1} , 1002 cm^{-1} , $1271\text{--}1355\text{ cm}^{-1}$ attributed to proteins was changed and for A549 cells addition to those peaks, the peak intensities at 838 cm^{-1} , 882 cm^{-1} , 1012 cm^{-1} , 1030 cm^{-1} attributed to proteins was also changed. In PNT1A cells,

the same changes with A549 cells were found with significant changes in the peak intensities. Similar to CytoD treatment, with Scr treatment, the protein profile change can be observed with new peaks appeared at 623 cm^{-1} , and 1155 cm^{-1} 1180 cm^{-1} , and 1190 cm^{-1} . With Scr treatment, dispersion of clathrin lattices are blocked which also hinders clathrin coated vesicle formation and vesicle scission. Moreover it is known that, when clathrin coated vesicle formation is blocked, the nucleation complex including adaptor protein and accessory proteins cannot disassemble and clathrin is not recruited and thus, vesicle does not form [434,435]. Until the nucleation step, adaptor protein AP2 and accessory proteins provide membrane invagination, clathrin coated pit formation and interaction with cargo. In that step, AP2 interacts with PIP2, phosphatidylinositol (3,4,5)-trisphosphate (PIP3) [134] and tyrosine based endocytosis motifs [135] to initiate clathrin coated pit formation. Moreover accessory proteins such as adaptin, amphipysin, endophilin, Eps15, intersectin are also used for clathrin polymerization and connection with actin cytoskeleton [140,141]. When clathrin polymerization was blocked by Scr, clathrin coated vesicle formation cannot finished and vesicle cannot bud from membrane, thus nucleation proteins stay still and maturation proteins cannot be recruited, and protein profile completely change. Moreover, with the decrease in the peak intensity at 501 cm^{-1} attributed to S-S and 1130 cm^{-1} attributed to phospholipids, it can be said that formation of the some of the endosomes are disrupted with Scr treatment.

The other observation is that the peak intensity at 548 cm^{-1} originating from cholesterol decreases while the intensity at 678 cm^{-1} attributed to ring breathing modes in the DNA bases increases in PNT1A cells with Scr treatment. These changes can be related to the side effects of the Scr. Although Scr is a commonly used inhibitor for the clathrin mediated endocytosis, it can cause cell shrinkage which results in the alterations of plasma membrane ion transport systems including pumps and channels [396]. Moreover, the induction of ion transporters activates kinases that may lead to the changes in the cortical actin cytoskeleton [389].

It was clearly observed that Scr treatment was cell type dependent and PNT1A cells were affected most. This can be explained with not only different AuNPs uptake of cells but also different Scr effect on them. The internalization of AuNPs was cell-type dependent as expected [34,209,436] and PNT1A cells internalize lesser amount of AuNPs and the highest amount of AuNPs were uptaken by A549 cells. Furthermore, as seen in flow

cytometry results in Figure 4.28., Scr treatment affected mostly PNT1A cells and most of the AuNPs could not enter the cells and thus could not provide SERS spectrum when compared to the other cells lines. Beas-2b cells were affected more than A549 cells, with the higher amount of internalized AuNPs; less effect of Scr was observed on SERS spectra. This change can also be explained with the protein corona formation. It is known that using different amount of protein source can alter the obtained intracellular SERS spectra [437]. Here, for each cell type, formed different protein corona, caused by the use of different media and different concentration of FBS, can affect not only the control group SERS spectra but also the SERS spectra obtained with Scr treatment as shown in the previous chapter.



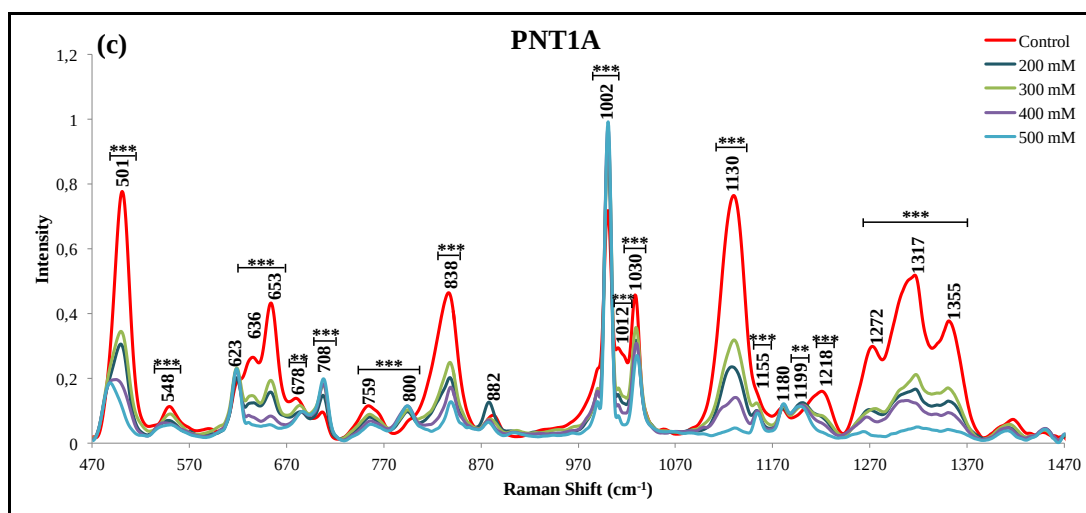


Figure 4.38. Average SERS spectra of (a) Beas-2b, (b) A549, and (c) PNT1A cells treated with Scr. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$ with the comparison of 500 mM Scr and control group

Figure 4.39. shows average SERS spectra PNT1A cell line treated with Cpm. As being ClMe inhibitor similar with Scr, Cpm provided similar significant changes in the SERS spectra with Scr treatment. Intensity decrease were observed at the peaks corresponding to endosomal membrane structures such as cholesterol (548 cm^{-1}), 1130 cm^{-1} (Phospholipid) which is related with the elimination of the ClMe for the PNT1A cell. When ClME was eliminated by Cpm, AuNPs enters the PNT1A cells via CaME and micropinocytosis. Thus, number of formed endosomes reduced which affects the obtained SERS spectra as decreased peak intensities corresponding to endosome membrane structures. Moreover, peak intensities at 708 cm^{-1} , 1002 cm^{-1} , attributed to proteins increased while at 838 cm^{-1} , 1012 cm^{-1} , 1030 cm^{-1} , $1271\text{-}1355 \text{ cm}^{-1}$ decreased. Moreover, protein profile changes were also observed with the peak intensity shifts at 623 cm^{-1} , $636/653 \text{ cm}^{-1}$ and 1155 , 1180 , $1999/1218 \text{ cm}^{-1}$ regions. Changed protein amount and profile is also related with the elimination of ClME by Cpm but also with Cpm action mechanism. Cpm causes loss of clathrin and AP2 adaptor complex on the cellular membrane which is the initiation molecules of ClME. Even AuNPs interact with the cell surface for the ClME, endosomal development could not be achieved and AuNPs could enter to the cells by CaMe of micropinocytosis. Thus, obtained peak intensities were found as decreased due to the inhibition.

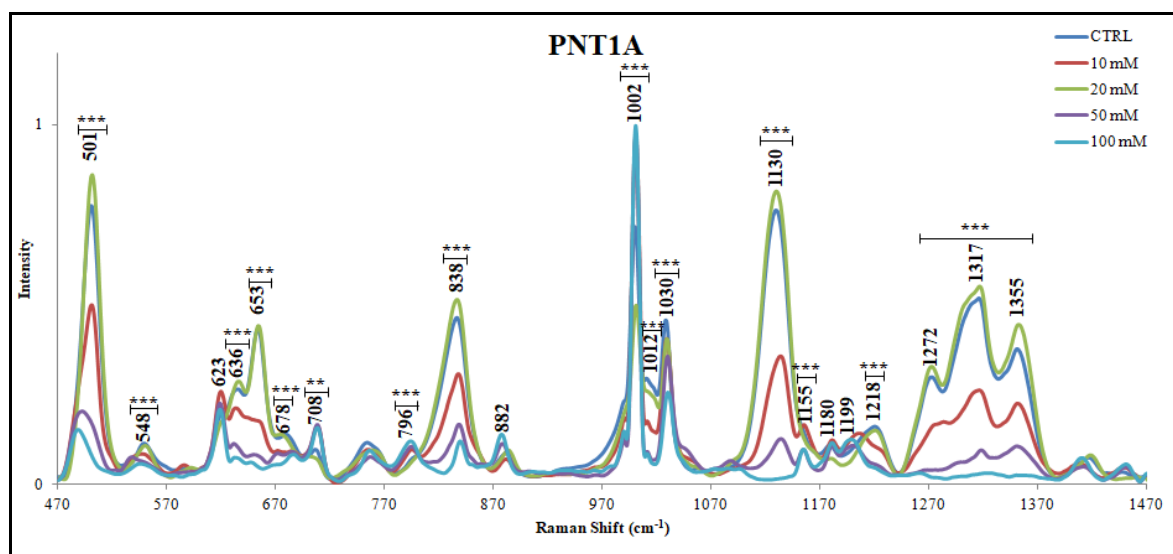


Figure 4.39. Average SERS spectra of PNT1A cells treated with Cpm. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$ with the comparison of 100 mM Cpm and control group

4.3.3.4. Statistical Analysis

For the observation of the variation of the SERS spectra, PCA and LDA was applied to the obtained average SERS spectra and obtained LDA plots for Scr treatment can be seen in Figure 4.40. As seen, For Scr treatment, better separation was achieved for three cell lines with increasing concentrations of Scr from control group. Obtained separation shows that Scr treatment provided significant spectral changes and this shows that most of the AuNPs reside inside the endosomes and these endosomes are different based on different pathways. In the point of Scr treatment, some part of the AuNPs were found in CME as cell type dependent manner and with Scr treatment changes in the CME can be tracked by SERS. Consequently, different endosomal pathway choices of the AuNPs could be interpreted by SERS and analyzed by LDA.

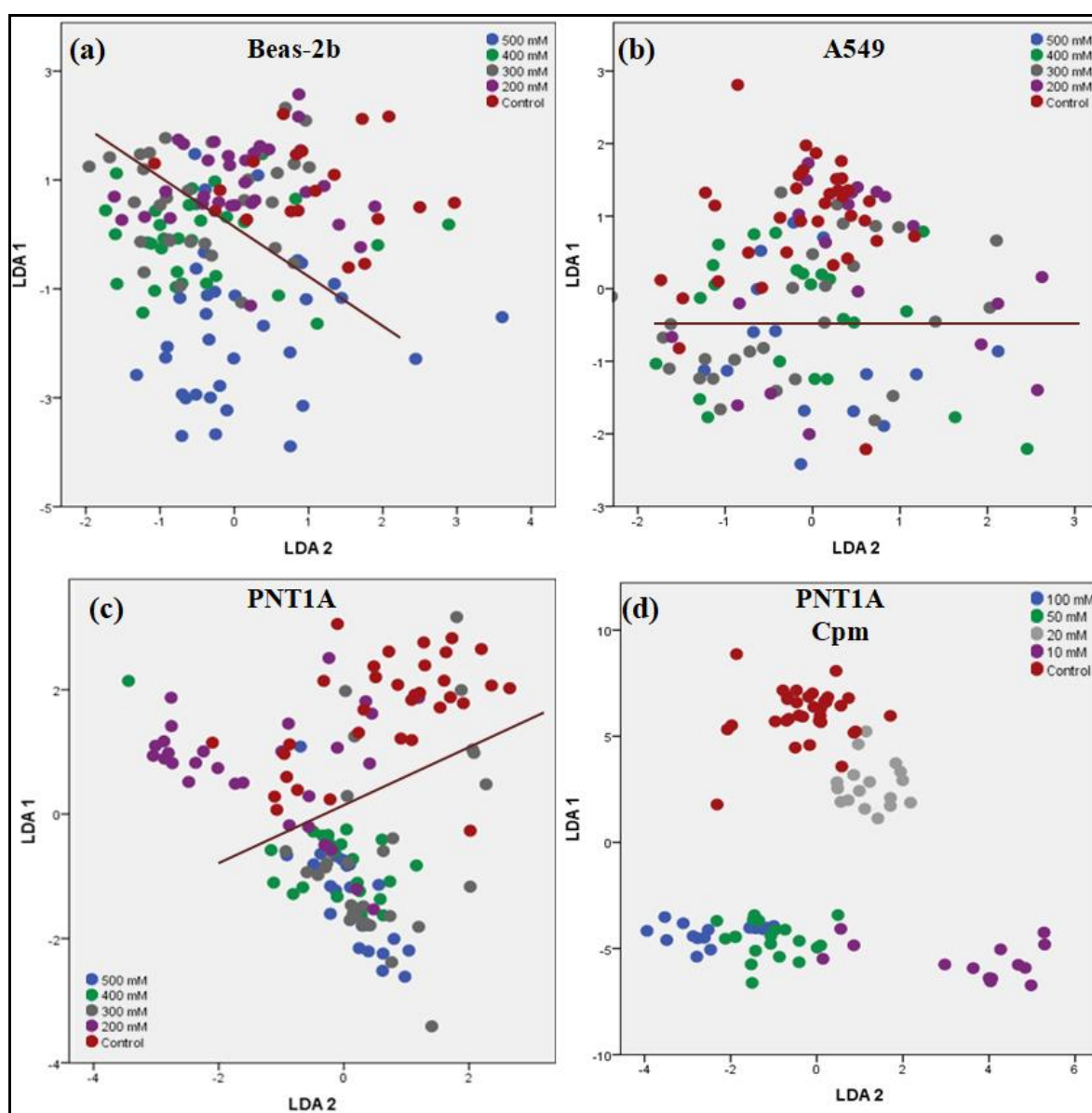


Figure 4.40. LDA Plots of (a) Beas-2b, (b) A549, (c) PNT1A cells treated with Scr and (d) PNT1A cells treated with Cpm

4.3.4. Inhibition of Caveolae Mediated Endocytosis

Caveolae mediated endocytosis of AuNPs were inhibited by using nystatin (Nys) and filipin (Fil) inhibitors. Fil and Nys are polyene antibiotics that affect cholesterol-rich domains. Fil and Nys provide formation of large aggregates in the membrane which accumulate and separate cholesterol from membrane. With that affect, cholesterol rich domains of the plasma membrane deform and this causes changes in caveolae and GPI-

anchored proteins which is ended up the inhibition of lipid raft ligands internalization [389,398-400].

4.3.4.1. Cytotoxicity of Nys and Fil

WST-8 cell proliferation results of A549, Beas-2b, HeLa and PNT1A cells treated with 2 h Nys and Fil were given in Figure 4.41. As seen, Fil did not cause any dramatic toxicity on cell lines although Nys caused dose dependent cell viability reduction on PNT1A cell line up to 80 percent.

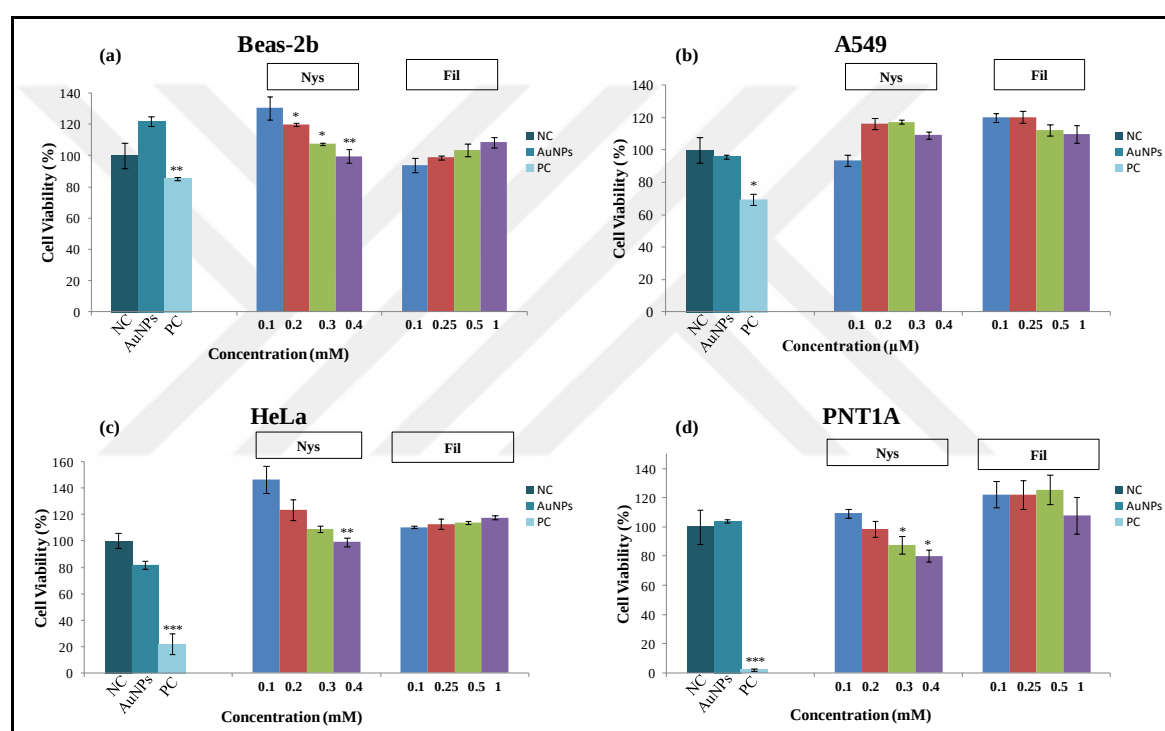


Figure 4.41. WST-8 Cell Proliferation results of (a) Beas-2b, (b) A549, (c) HeLa, and (d) PNT1A after 2 h exposure to Nys or Fil. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$. PC: Positive control (10 percent DMSO was used as positive control), NC: Negative control

4.3.4.2. Uptake Inhibition by Nys and Fil

Caveolae dependent endocytosis was inhibited by the use of Nys and Fil and inhibition rates were observed by investigation of SSC decrease as seen in Figure 4.42. Nys caused AuNPs internalization decrease up to 56 percent on Beas-2b, 35 percent on HeLa and 98 percent on PNT1A cells. For Fil treatment, AuNPs uptake decrease was found as 89 percent for Beas-2b, 95 percent for A549 and 90 percent for PNT1A cell line. Consequently, Nys affected all cell lines and Fil affected them except for HeLa cells which mean that all cell types prefer caveolae mediated endocytosis for the internalization of approximately 50 nm AuNPs and HeLa cells were affected by Nys and Fil differentially due to their mechanisms of action.

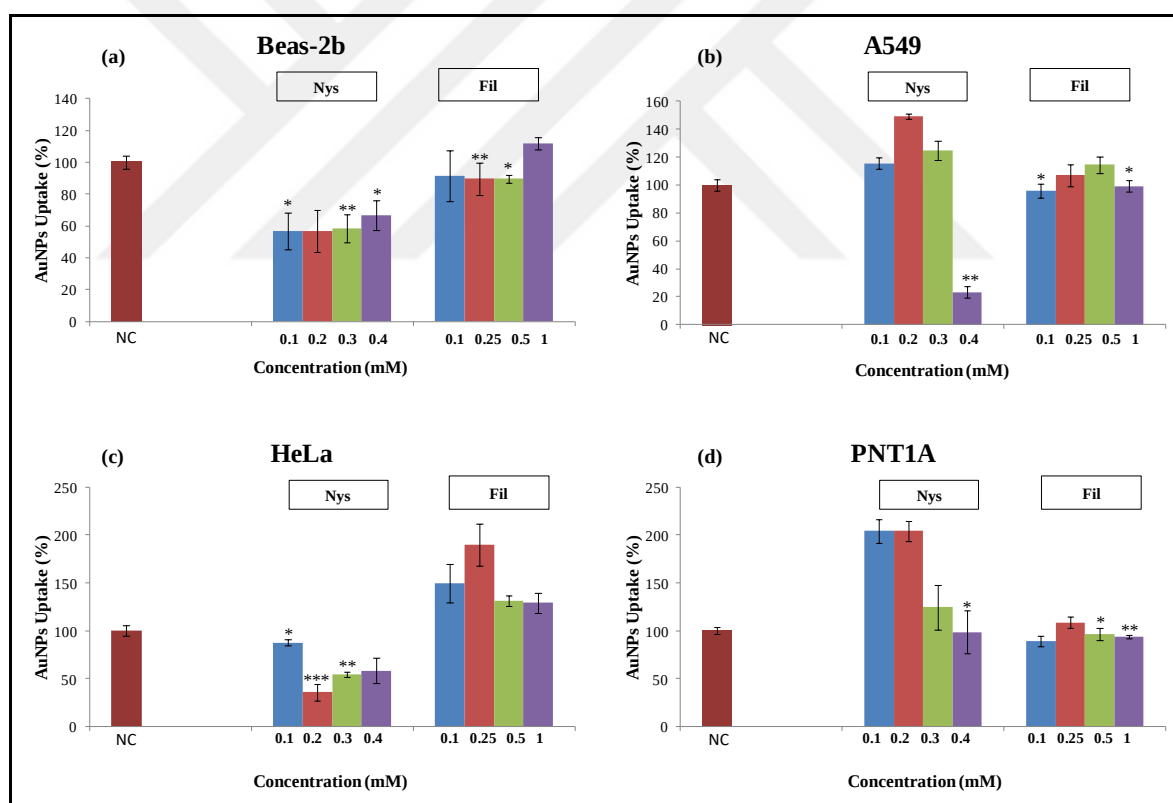


Figure 4.42. Flow cytometry analysis of a) Beas-2b, b) A549, c) HeLa, and d) PNT1A after 2 h exposure to Nys or Fil then 22 h AuNPs. NC: Negative control

4.3.4.3. *SERS Measurements*

Caveolae mediated endocytosis was inhibited by Nys and Fil. Fil and Nys provide formation of large aggregates in the membrane which accumulate and separate cholesterol from membrane [389,398-400].

SERS spectra of Fil treated Beas-2b, A549, and PNT1A cells are shown in Figure 4.43. As seen, the healthy cell lines PNT1A and Beas-2b were more affected than cancerous A549 cell line. Moreover, similar to the Scr treatment, most effected cell line is PNT1A and this change can also be explained with the protein corona formation as in the Scr treatment. Formed different type of protein corona can alter the obtained SERS spectra with Fil treatment. Thus, differential effect of the Fil is caused by not only the cell type dependent effect mechanism of the used inhibitor but also the media and protein source dependent protein corona formation. When the cells were treated with Fil, for Beas-2b and PNT1A cells, mostly protein profile change was observed with appeared new peaks at 623 cm^{-1} , 1155 cm^{-1} , 1180 cm^{-1} and 1199 cm^{-1} and the decrease of the peak intensity at 656 cm^{-1} and 1212 cm^{-1} . Furthermore, peak intensities at 501 cm^{-1} (S-S vibrational peak), 678 cm^{-1} (DNA bases), 708 cm^{-1} (C-S), 800 cm^{-1} (Phosphate ion interactions), 838 cm^{-1} (Tyr), 882 cm^{-1} (protein), 1002 cm^{-1} (Tyr), 1012 cm^{-1} (Trp), 1130 cm^{-1} (phospholipids), and $1275\text{--}1350\text{ cm}^{-1}$ (Amide III) were found to be varied due to the disruption of CaME. With the Fil treatment, cholesterol is separated from plasma membrane and caveolae mediated endocytosis cannot be initiated from the lipid raft domains. Thus, AuNPs can only be internalized by other endocytosis pathways to the cells causing protein profile changes and decrease in the intensity of the peaks attributed to phospholipid on the SERS spectrum. For A549 cells, SERS spectrum does not change much with Fil treatment; however protein profile change can be observed from the peaks at $623\text{--}656\text{ cm}^{-1}$ and $1180\text{--}1218\text{ cm}^{-1}$. As a result, the spectral changes originating from Fil treatment can be observed with SERS even there is a slight change in the AuNPs internalization with Fil treatment.

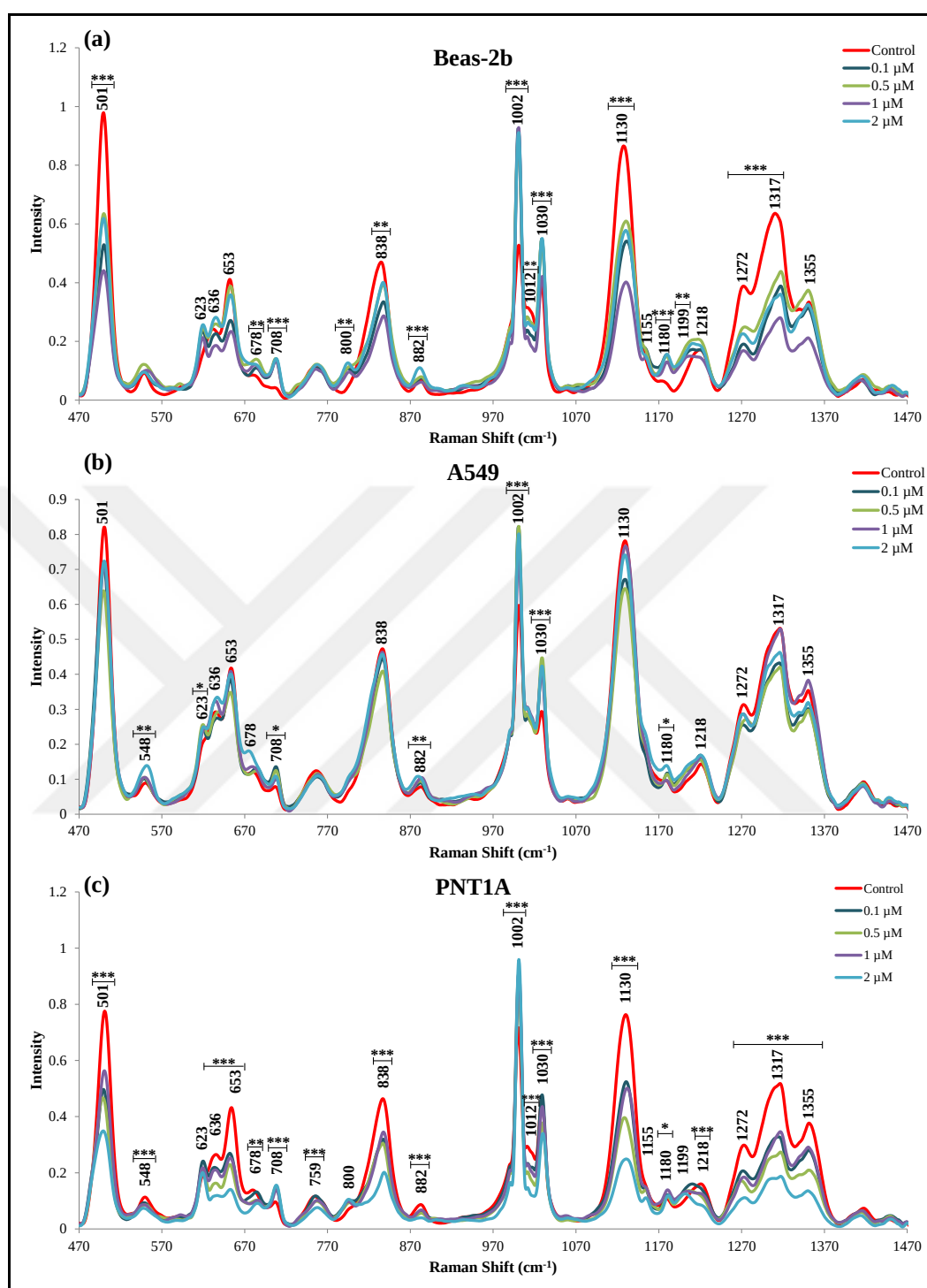
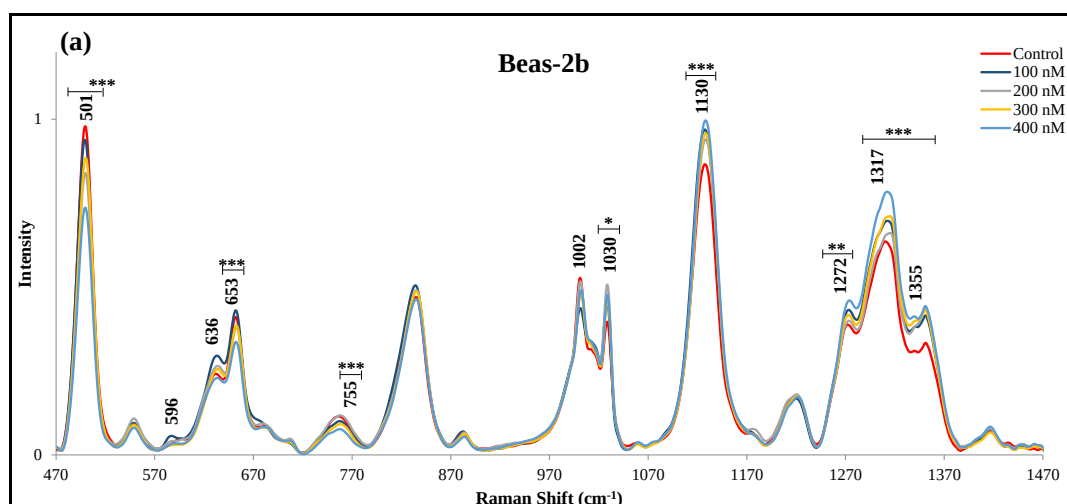


Figure 4.43. Average SERS spectra of (a) Beas-2b, (b) A549, and (c) PNT1A cells treated with Fil. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$ with the comparison of 2 μM Fil and control group

SERS spectra of Nys treated Beas-2b, HeLa, and PNT1A cells are shown in Figure 4.44. As seen, Nys caused few spectral changes when compared with Fil treatment. Fil was found more affected when compared with Nys which can be related with the side effects of Fil. Although working principle of Nys and Fil are almost similar, Fil affects cells more due to the disruption of cell permeability which is caused by the undesired interaction of Fil with membrane phospholipids and disruptions of the F-actin linkage and plasma membrane [438,439].

As seen, peak intensities at 501 cm^{-1} (S-S), 653 cm^{-1} (Tyr), 1030 cm^{-1} (Phe), 1130 cm^{-1} (Phospholipid), $1272\text{--}1352\text{ cm}^{-1}$ (Amide III) changed significantly. Even changes in the peak intensities were slight, same peaks changes were observed with the Fil treatment due to the inhibition of the same endocytosis pathway. Like Fil, Nys removes cholesterol from lipid rafts. With the removal of cholesterol, CaME is blocked, less endosomes were formed and obtained peak intensity at 501 cm^{-1} and 1130 cm^{-1} , which are originated from the endosomes, is reduced. On the other hand, remaining intensity changes were observed at the peaks originating from proteins due to the altered protein profile near or on the AuNPs in the endosomes by inhibition. It is known that, with cholesterol depletion, caveolin1 is flattened and mobility of caveolin1 in the membrane increases. With the depletion of caveolin1 due to the increased mobility, activated Cdc42 levels increase [440,441]. On the other hand, with the removal of cholesterol, CaME could not be initiated and proteins found near or on the AuNPs for the internalization could not be recruited to the site and maturation could not be achieved which requires protein recycling and recruitment. Thus, with the inhibition process, protein profile is changed, and this can be observed in the SERS spectra as slight changes in the peak intensities originating from proteins.



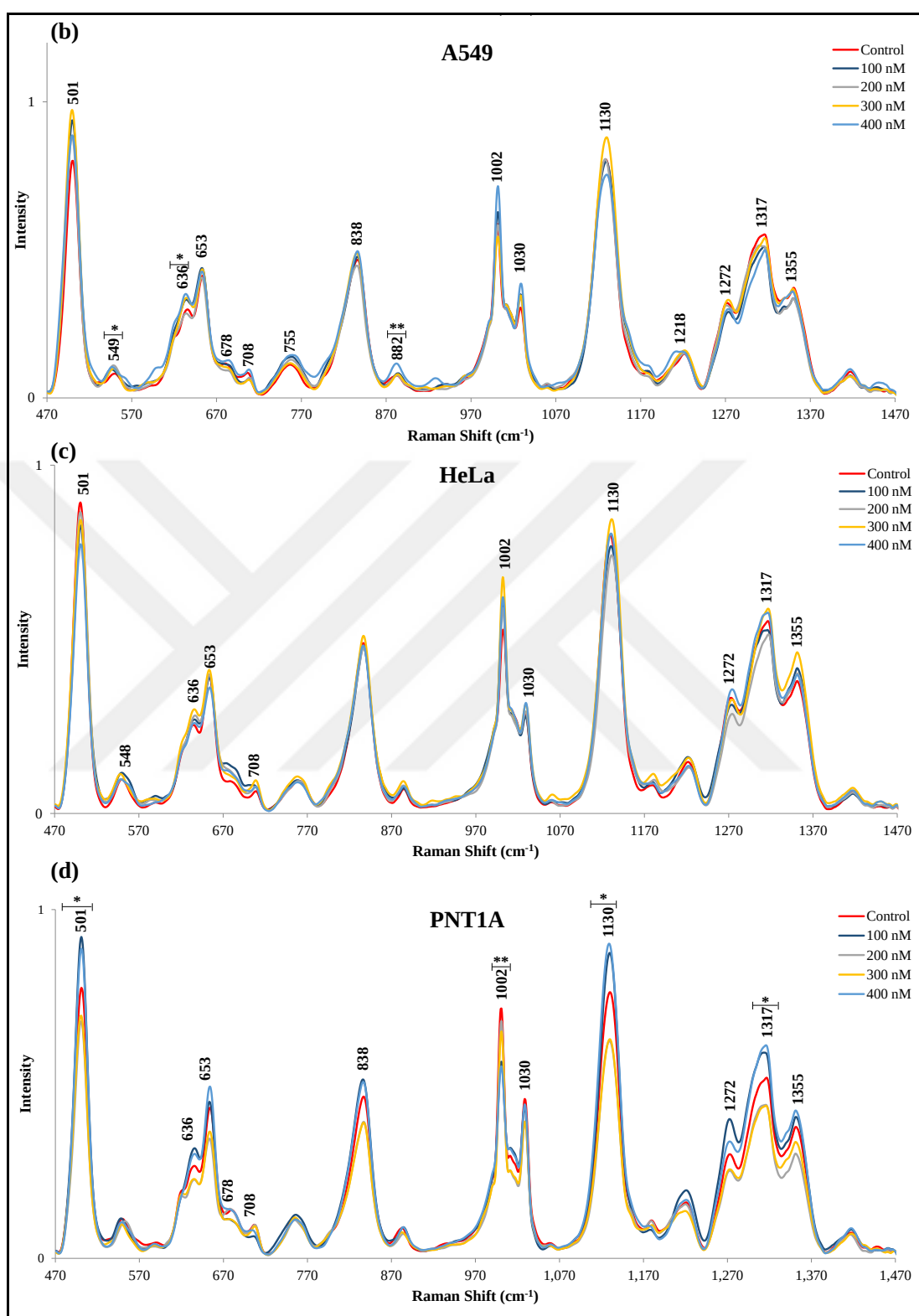


Figure 4.44. Average SERS spectra of (a) Beas-2b, (b) A549, (c) HeLa, and (d) PNT1A cells treated with Nys. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$ with the comparison of 400 nM Nys and control group

4.3.4.4. Statistical Analysis

For the observation of the variation of the SERS spectra, PCA and LDA was applied to the obtained average cellular SERS spectra after Nys and Fil treatment and obtained LDA plots for Nys and Scr treatment can be seen in Figure 4.45. and 4.46. For Nys treatment, not a good separation was achieved for lower concentrations although highest concentrations were completely separated. When cell type dependent effect was considered, best separation was achieved for PNTA cell line. For the Fil treatment, like Nys treatment, highest concentrations provide better separation when compared with lower concentrations and similar separation patterns were observed for all cell lines. When compared two inhibitors, Fil treatment provide better separation of treatment groups from control group. Better separation means that with Fil and Nys treatment, CaME is inhibited and inhibition was reflected to the SERS spectra significantly. This also shows that AuNPs are uptaken to the cells by CaME as cell type dependent manner and inhibition of CaME can be tracked by SERS significantly.

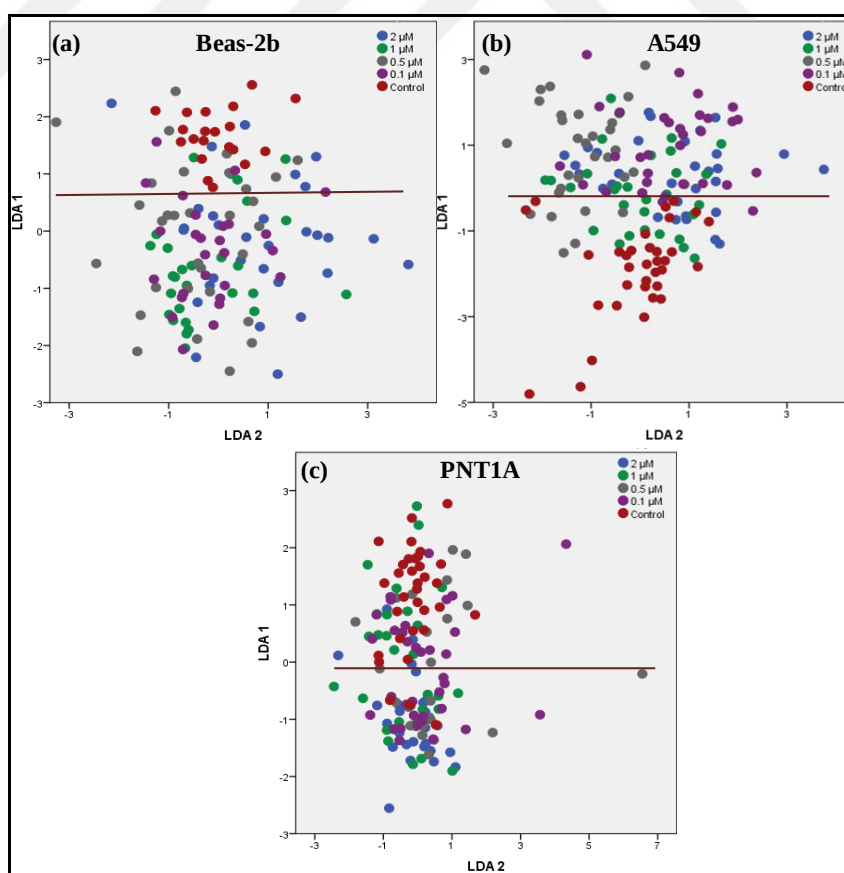


Figure 4.45. LDA Plots of (a) Beas-2b, (b) A549, and (c) PNT1A cells treated with Fil

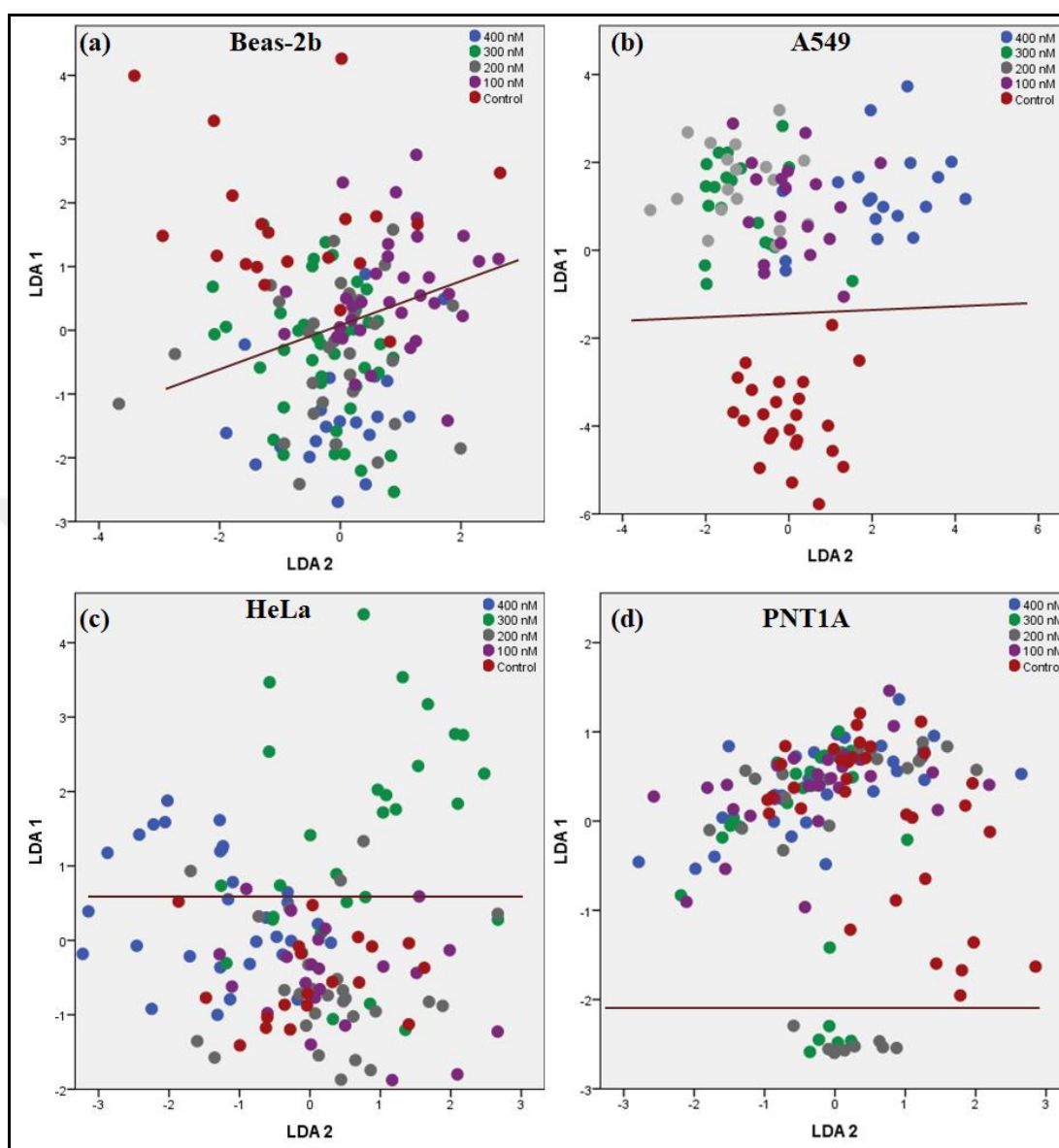


Figure 4.46. LDA Plots of (a) Beas-2b, (b) A549, and (c) PNT1A cells treated with Nys

4.3.5. Inhibition of Dynamin Dependent Endocytosis (DDE)

For the investigation of dynamin dependent endocytosis (DDE), dynasore (Dyn) was used to block vesicle scission by the inhibition of dynamin protein. With the inhibition of dynamin protein both clathrin and caveolae mediated endocytosis can be eliminated for the uptake of AuNPs, because dynamin protein has role in both endocytosis pathways that helps vesicle scission and formation of early endosomes. Furthermore, among the used

inhibitors dynasore is the most specific agent that is used for the inhibition of endocytosis pathway.

4.3.5.1. Cytotoxicity of Dyn

WST-8 Cell Proliferation results of A549, Beas-2b, HeLa and PNT1A cells treated with 2 h Dyn were given in Figure 4.47. As seen, Dyn did not cause any significant toxicity on any cell types.

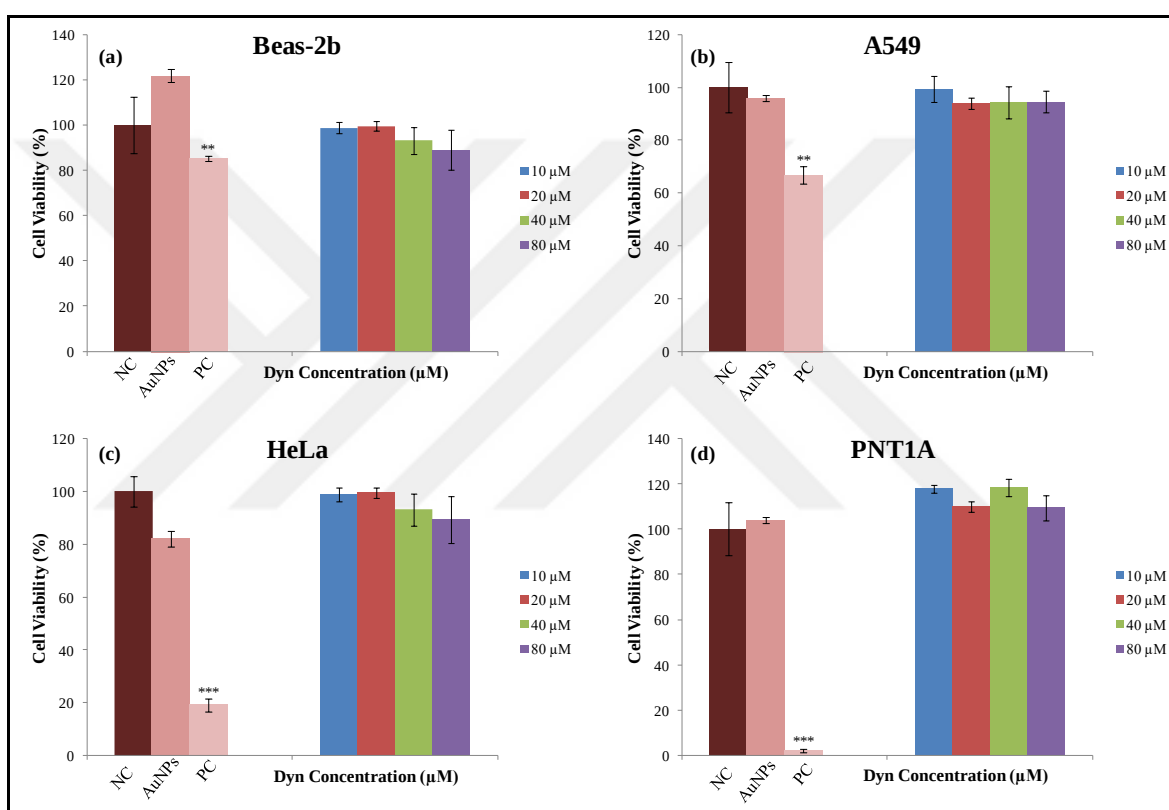


Figure 4.47. WST-8 Cell Proliferation results of (a) Beas-2b, (b) A549, (c) HeLa, and (d) PNT1A after 2 h exposure to dynasore. PC: Positive control (10 percent DMSO was used as positive control), NC: Negative control. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$ with the comparison of 400 nM Nys and control group

4.3.5.2. Uptake Inhibition by Dyn

Clathrin and caveolae mediated endocytosis pathways were inhibited by the use of Dyn and inhibition was examined using flow cytometry. According to the obtained results shown in Figure 4.48., inhibition of dynamin dependent endocytosis can be observed with

Dyn treatment. Thanks to being the most specific inhibitor used for the endocytosis inhibition, higher than 100 percent SSC shift was not observed with Dyn treatment. While all used inhibitors cause side effects on cellular mechanisms, Dyn does not cause any side effects on cellular mechanisms and provide unique interaction with only dynamin protein. With a specific interaction, Dyn did not cause dramatic decreases in AuNPs uptake although caused SSC decrease significantly in Beas-2b cells up to 70 percent, in A549 cells up to 79 percent and in HeLa cells up to 66 percent. Dyn could not provide any AuNPs uptake decrease in PNT1A cell.

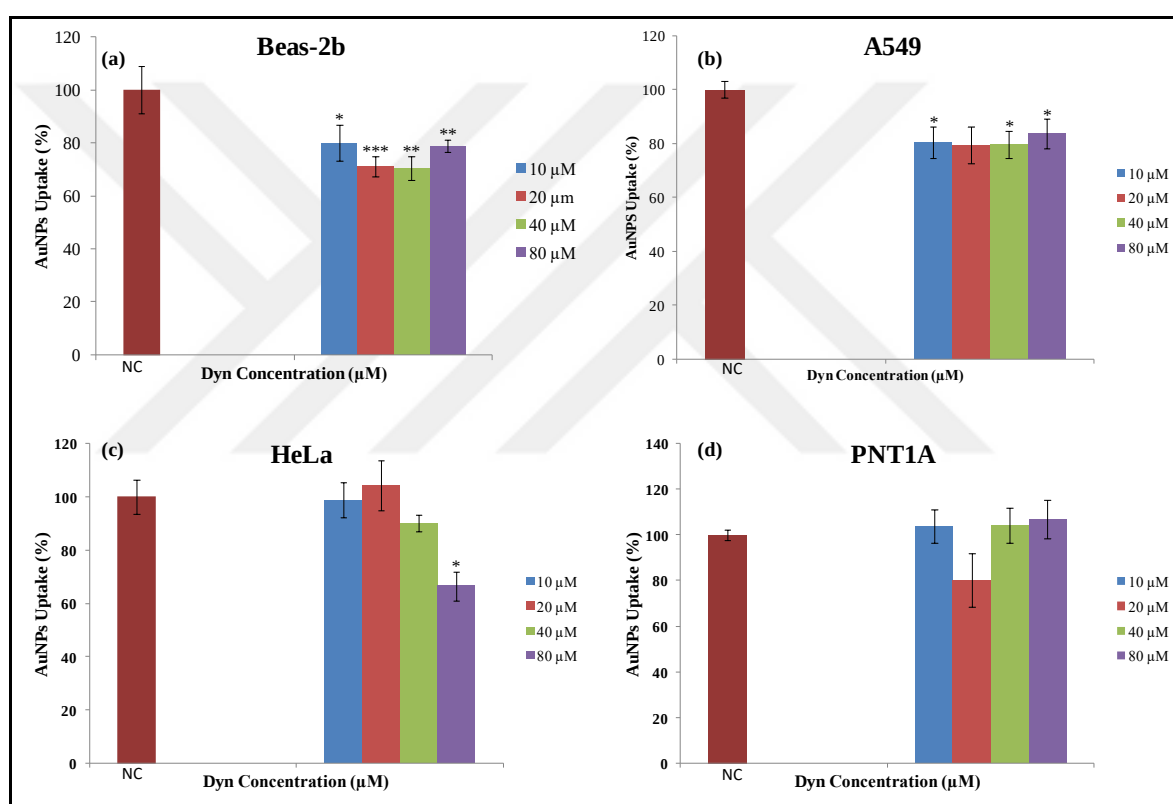


Figure 4.48. Flow cytometry analysis of a) Beas-2b, b) A549, c) HeLa, and d) PNT1A after 2 h exposure to dynasore then 22 h AuNPs. NC: Negative control. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$ with the comparison of 400 nM Nys and control group

4.3.5.3. SERS Measurements

For the dynamin dependent endocytosis inhibition, Dyn treated SERS spectra of Beas-2b, A549 and HeLa cells are shown in Figure 4.49. When Dyn was used as dynamin dependent endocytosis inhibitor, not dramatic changes compared with other treatments were obtained. This is caused by the mechanisms of used inhibitors, while other types of inhibitors caused many side effects on cellular metabolisms and provide more changes, Dyn could only block dynamin protein in the endocytosis pathway and not cause any side effects. This could be the reason of obtained slight differences. When the obtained cellular SERS spectra are considered, for Beas-2b cell line, peak intensities at 501 cm^{-1} (S-S), 708 cm^{-1} (C-S), 1180 cm^{-1} (Tyr, Phe) were found as changed significantly. For A549 cell line, only one peak intensity was changed as increase at 708 cm^{-1} (C-S). For HeLa cell line, more peak intensity changes were observed at 501 cm^{-1} (S-S), 623 cm^{-1} (C-C), 708 cm^{-1} (C-S), 882 cm^{-1} (Protein), and 1130 cm^{-1} (Phospholipid). All the intensity changes were originating from either endosomal structure such as 501 cm^{-1} caused by the formation of endosomes that cystein rich proteins play a role and 1130 cm^{-1} originating from phospholipids or proteins such as 623 , 708 , 882 and 1180 cm^{-1} due to the disruption of dynamin dependent endocytosis pathways. Even obtained changes were slight; they can be correlated with the changes in the endosomal pathway in the presence of Dyn. With the use of Dyn, dynamin protein could not bound to the neck of endosome and provide scission of the endosome to initiate early endosome formation [403]. Endosomes could not be matured and fuse with lysosomes in the case of ClME and also could not recycled back in the case of CaME. Thus, two types of the endocytosis pathways were disrupted and peak intensities correlated with endocytosis decreased and due to the protein profile change, peak intensities originating from proteins changed. When the cell type dependent changes were considered, most peak intensity changes were observed at HeLa cell line and least changes were observed at A549 cell line. These results could be explained with the cellular uptake rate of AuNPs in the presence of Dyn as seen in Figure 4.48. As seen, most AuNPs decrease was also achieved by HeLa cell line with uptake reduction up to 66 percent while Dyn decreased AuNPs uptake of Beas-2b cell line up to 70 percent and A549 cell uptake rate decreased up to 79 percent. With the alterations in the decrease rate, not only amount of AuNPs internalized changes, but also obtained SERS signals changes. When HeLa cells

had lower number of AuNPs inside the cells, the signal changes observed more with the Dyn treatment.

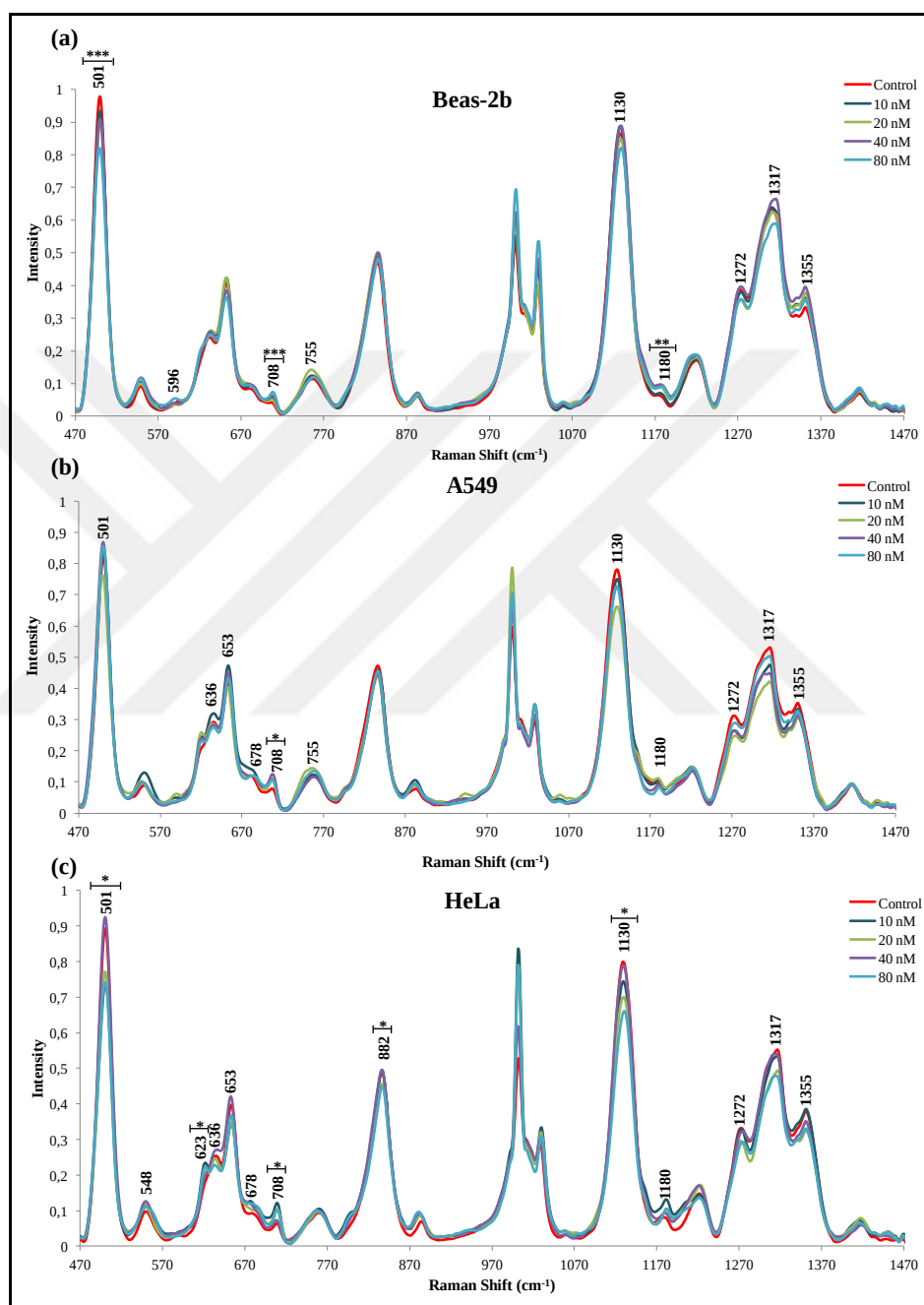


Figure 4.49. Average SERS spectra of (a) Beas-2b, (b) A549, and (c) PNT1A cells treated with Dyn. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$ with the comparison of 80 nM Dyn and control group

4.3.5.4. Statistical Analysis

For the observation of the variation of the SERS spectra, PCA and LDA was applied to the obtained average cellular SERS spectra after Dyn treatment and obtained LDA plots can be seen in Figure 4.50. As seen, not a quiet separation was achieved due to the small effect of the Dyn on the cells without any disruption of other cellular metabolisms as seen in other inhibitors.

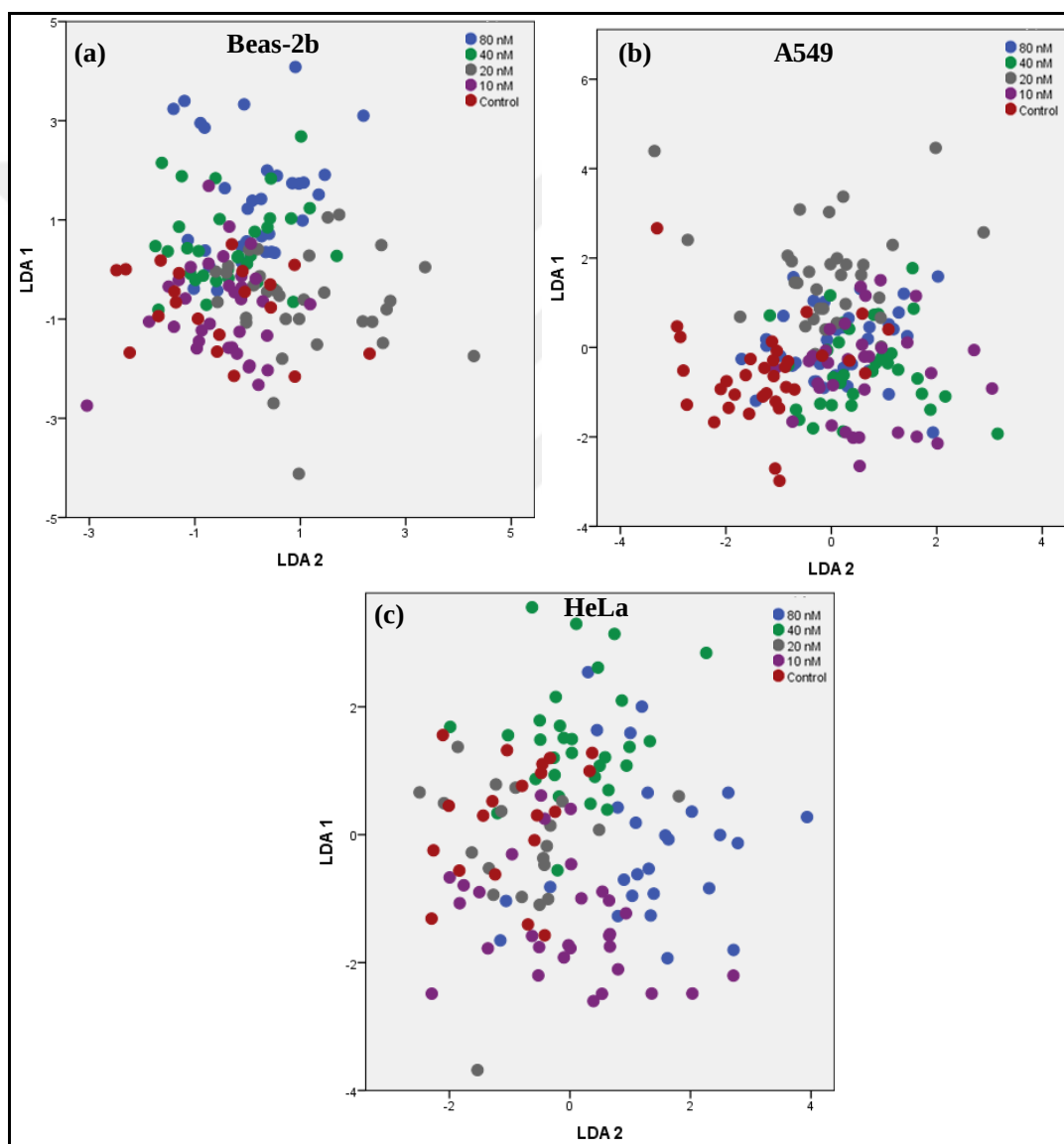


Figure 4.50. LDA Plots of (a) Beas-2b, (b) A549, and (c) PNT1A cells treated with Dyn

4.3.6. Inhibition of Macropinocytosis

Macropinocytosis was inhibited by CytoD and Aml. CytoD block actin polymerization by occupying fast-growing (barbed) ends of actin filaments and also interacting with capping proteins (CAPZA1 and capping protein α subunit family) [420]. Aml is an inhibitor of Na^+/H^+ exchangers, which hinders the elimination of intracellular excess H^+ and this is resulted in the intracellular impaired acidification and disruption of macropinocytosis [423,424].

4.3.6.1. Cytotoxicity of CytoD and Aml

WST-8 cell proliferation results of A549, Beas-2b, HeLa and PNT1A cells treated with CytoD or Aml are given in Figure 4.51. As seen, inhibitors do not cause any significant cytotoxicity on the four cell lines.

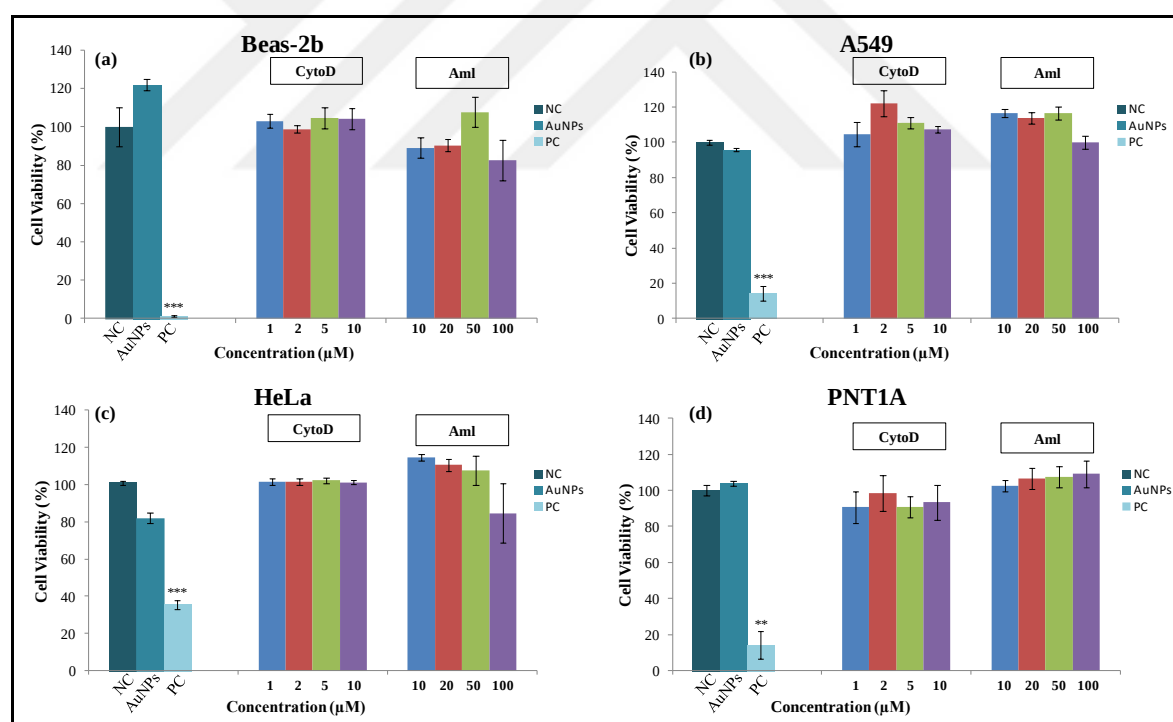


Figure 4.51. WST-8 Cell Proliferation results of (a) Beas-2b, (b) A549, (c) HeLa, and (d) PNT1A treated with CytoD and Aml. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$. PC: Positive control (10 percent DMSO was used as positive control), NC: Negative control

4.3.6.2. Uptake Inhibition by CytoD and Aml

The inhibition of the AuNPs uptake in the presence of macropinocytosis inhibitors were investigated by flow cytometry and shown in Figure 4.52. As seen, CytoD caused significant uptake decrease in most of the doses for Beas-2b cell line up to 55 percent, and in the highest dose for A549 cell line up to 91 percent. Aml caused dramatic AuNPs uptake decrease in Beas-2b up to 19 percent and in PNT1A up to 15 percent. Both inhibitors did not cause any inhibition of AuNPs uptake for HeLa cells. Consequently, it can be said that Beas-2b, A549 and PNT1A cells choose macropinocytosis for the AuNPs internalization but not HeLa cells.

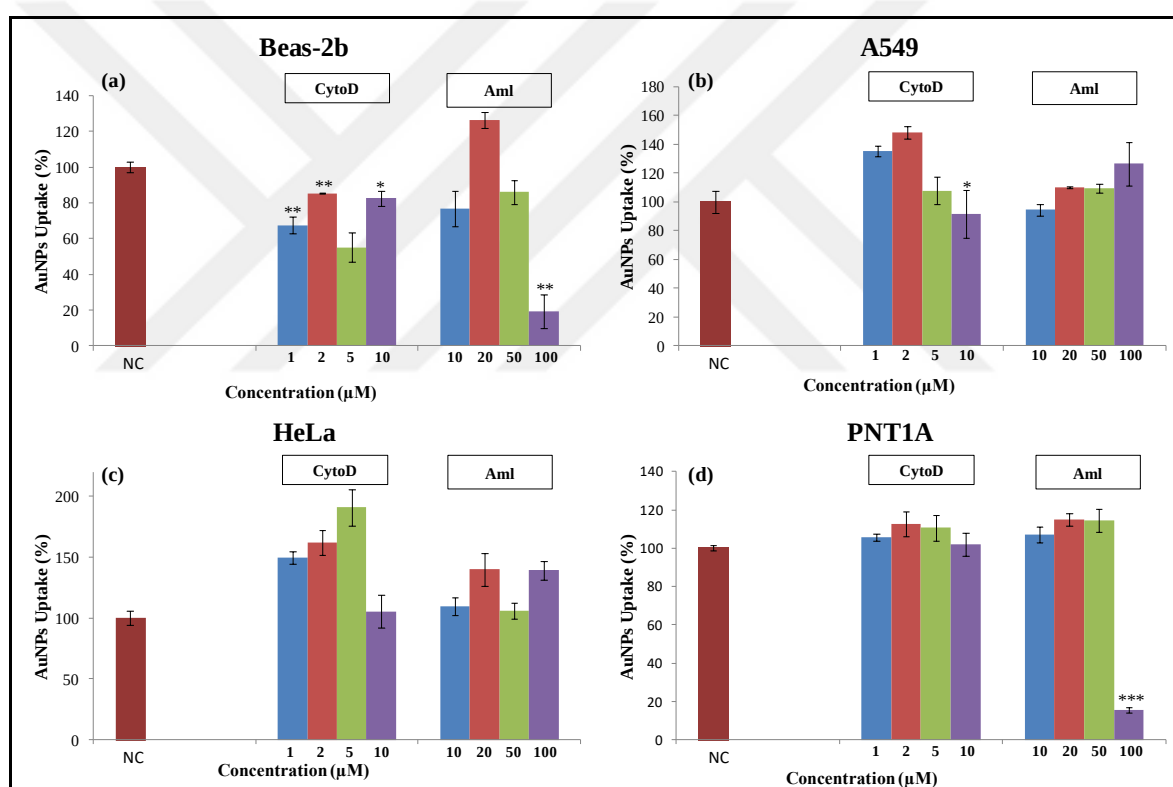


Figure 4.52. Flow cytometry analysis of a) Beas-2b, b) A549, c) HeLa, and d) PNT1A cells after 2 h exposure to CytoD and Aml then 22 h AuNPs. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$. NC: Negative control

4.3.6.3. *SERS Measurements*

For the inhibition of macropinocytosis, CytoD was used for the blockage of actin polymerization which prevents membrane ruffling and macropinocytosis. CytoD block actin polymerization by occupying fast-growing (barbed) ends of actin filaments and also interacting with capping proteins (CAPZA1 and capping protein α subunit family) [420]. Actin polymerization is blocked by CytoD with two major steps. In the nucleation step, it binds to the barbed ends of actin where oligomerization reaction cannot occur, in elongation step elongation cannot be obtained while barbed ends are occupied by CytoD [421]. Furthermore, CytoD can also affect the ClME due to the role of actin in also ClME [422]. In CaME actin does not play any role, however recycling of the endosomes back to the plasma membrane is also regulated by actin [171].

When Beas-2b and A549 cells were treated with increasing concentrations of CytoD, it was found that almost same peak changes were observed for both of the cell lines as shown in Figure. 4.54. When spectral changes are interpreted, molecular environment where AuNPs found in endosomes should be considered. Molecular environment of the AuNPs during micropinocytosis is illustrated in Figure 4.53. As shown, during the micropinocytosis, cargo which is AuNPs in our cases found in macropinosomes interacted with the macropinosome membrane. During the development of the pathway, many different molecules including proteins and PIs are recruited to the site and with the maturation for the new molecule recruitment, recruited molecules are dissociated from the cite. During this development and also during the inhibition of micropinocytosis, AuNPs reside inside the endosomes but affected from all type of molecules changes in the surrounding environment because all events happen in a few nanometers far from the AuNPs. Thus, pathway development steps and also inhibition mechanisms of the used inhibitors could be used for the interpretation of the obtained spectral changes.

For both Beas-2b and A549 cell lines, the peak intensity corresponding to S-S bond (501 cm^{-1}) decreased significantly due to the disrupted macropinocytosis. When macropinocytosis was blocked by CytoD, AuNPs can enter to the cells via only ClME and CaME for both Beas-2b and A549 cells. However, it is also known that CytoD can affect the ClME due to the role of actin [422]. In CaME, actin does not play any role but recycling of the endosomes back to the plasma membrane is also regulated by actin [171].

On the other hand, it is known that increase in the peak intensity at 501 cm^{-1} caused by the formation of endosomes that cystein rich proteins play a role due to the strong bonding of the AuNPs with thiol groups in cysteine residues [442]. Thus, the obtained intensity was found as decreased when compared to the control.

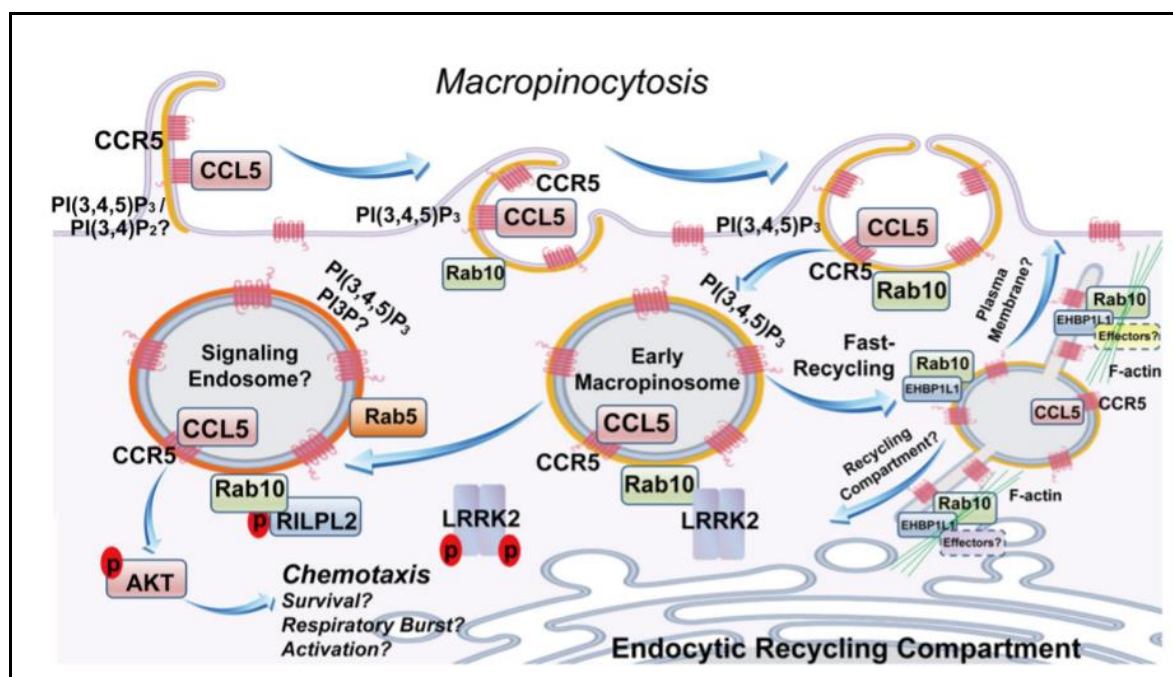


Figure 4.53. Schematic representation of macropinocytosis pathway [443]

Moreover, the peak intensities originating from proteins (708 , 755 , 838 , 1002 , 1012 , 1030 cm^{-1}) were found to be changed significantly due to the altered protein profile surrounding AuNPs during macropinocytosis as a result of CytoD treatment. The significant spectral pattern changes observed at 623 cm^{-1} / 636 cm^{-1} and 1155 , 1180 , 1199 and 1218 cm^{-1} , which they fall into the protein fingerprint region. A new peak appears at 623 cm^{-1} while the intensity of peak at 636 cm^{-1} decreases significantly. Three new peaks are observed at 1155 , 1180 and 1199 cm^{-1} while the peak intensity at 1218 cm^{-1} decreased significantly with the treatment of increased concentrations of CytoD. These spectral changes can be related to the changed protein profile during endocytosis with CytoD treatment. In macropinocytosis, actin involvement starts with the movement of formed macropinosomes from the membrane [187]. At this stage, many types of proteins are used for the regulation and development of macropinocytosis. With Arp2/3 complex activation and recruitment of WASP-interacting Protein (WIP) type I myosins, Wiskott–Aldrich Syndrome protein

(WASP) starts to nucleate actin filaments. Actin initiation starts with interaction of WASP with CD2AP for its inactivation which promotes recruitment of syndapin to allow initiation of actin nucleation. For the regulation of actin nucleation, other proteins such as Bbc1p, mAbp1p and coronin have also roles. With the filament formation, membrane invagination and vesicle formation start. When vesicle buds out from the membrane, the regulatory proteins mentioned above disassembled from the endocytic site [188]. For the movement of the vesicle, actin bundles are used with the help of actin bundling proteins such as fimbrin. The stability of the obtained actin network was also sustained by proteins such as transgelin. After this step, macropinosome fuse with endosome and form late endosomes and then endolysosomes [195,196]. When CytoD is used, nucleation and elongation of actin cannot be obtained, some of the regulatory proteins, which should disassemble after scission of vesicle, cannot disassemble from the vesicle and some of the regulatory proteins responsible in the later steps of the macropinocytosis such as movement of the macropinosome, fusion with endosome, formation of late endosomes, etc. cannot be recruited to the endocytic site. Due to these changes, protein profile near or on the AuNPs which are entrapped in endosomes are changed and this is reflected on the SERS spectra.

Furthermore, it was found that peak intensity at 800 cm^{-1} which is attributed to phosphate ion interactions increased with CytoD treatment. This change can be related to the changed phosphatidylinositol(4,5)biphosphate (PIP_2) mechanism with disrupted macropinocytosis. PIP_2 is used for the regulation of the endocytic pathway for whole process [444]. In the early steps of the endocytosis, PIP_2 levels are increased with phosphorylation and it is required for the membrane invagination initiation and pit formation. Then, PIP_2 levels are decreased by phosphatases for the recruitment of regulatory proteins to the endocytic site required for the maturation of the endocytic process [58]. With the CytoD treatment, maturation of the macropinocytosis is disrupted and PIP_2 levels cannot be decreased by phosphatases via dephosphorylation and new regulatory proteins cannot recruit. This disrupted PIP_2 regulatory mechanism can be seen as peak intensity change at the 800 cm^{-1} in the SERS spectra.

The intensity of the peak at 1130 cm^{-1} attributed to phospholipids was found decreased significantly. Due to the elimination of the one of the internalizations pathways of AuNPs, less amount of AuNPs were internalized with only two type of endocytosis. Thus, lesser

number of endosomes was formed by cells and dependently the intensity of the peak attributed to phospholipid intensity decreased.

When the SERS spectra from Beas-2b and A549 cells were compared, it was realized that peak intensity changes were more dramatic for Beas-2b cells. This could be the result of the nature of these cell lines. Healthy Beas-2b cells were more affected by CytoD treatment while cancerous A549 were more resistant to the effects of selected inhibitor. The control group of Beas-2b cells were more separated than the control group of A549 cells which means CytoD treatment was more affective on Beas-2b cells.

On the other hand, when all inhibitor treatments were compared, most drastic changes in the obtained SERS spectra were found with CytoD treatment. The reason of this dramatic effect is possibly caused by the involvement of actin in not only macropinocytosis but also CaME [445] and ClME [173].

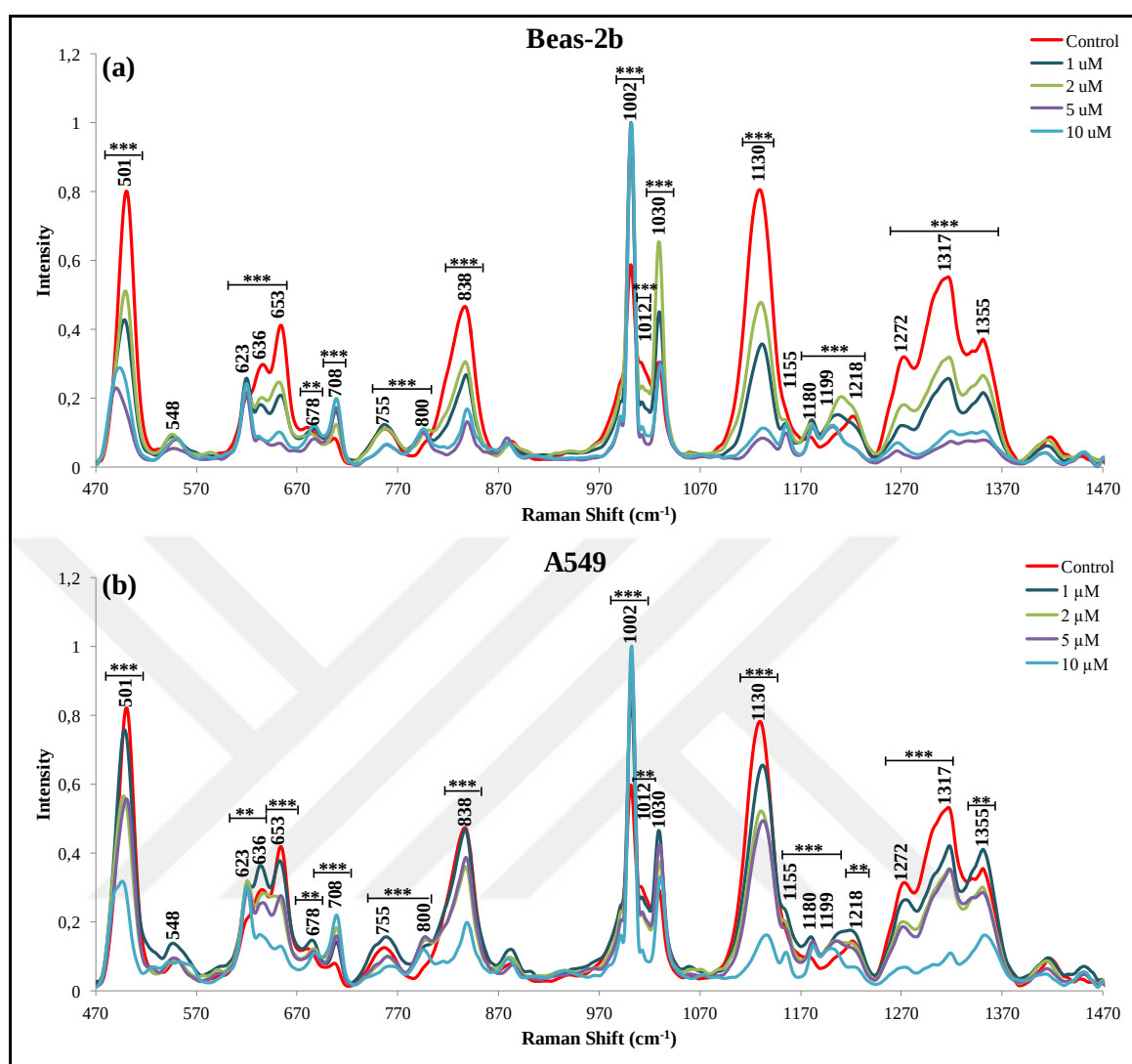


Figure 4.54. Average SERS spectra of (a) Beas-2b, (b) A549 cells treated with CytoD. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$ with the comparison of 10 μ CytoD and control group

As another inhibitor Aml was also used for the inhibition of macropinocytosis. Obtained SERS spectra belong to Beas-2b and PNT1A can be seen in Figure 4.55. For Beas-2b cells, almost same spectral changes were observed with CytoD treatment. Peak intensities were found to decrease at 501 cm⁻¹ (S-S vibrational peak), 755 cm⁻¹ (Trp symmetric breathing), 838 cm⁻¹ (Tyr), 1012 cm⁻¹ (Trp), 1030 cm⁻¹ (Phenylalanine, C-N stretching), 1130 cm⁻¹ (phospholipids), 1218 cm⁻¹ (Protein) and some of them were found to increase at 708 cm⁻¹ (C-S), 800 cm⁻¹ (Phosphate ion interactions), 1002 cm⁻¹ (Tyr), 1180 cm⁻¹ (Tyr, Phe), and

1199 cm^{-1} (Trp) for both of the inhibitors. For PNT1A cells, significant changes could not be observed in the SERS spectra. Even 100 μM Aml caused 85 percent drop in the AuNPs uptake rate, due to having least amount of internalized AuNPs when compared with other cell lines, obtained spectra did not changed significantly. It should be also explained as that when PNT1A cells treated with different type of inhibitors, Scr treatment caused both significant uptake reduction and also significant spectral changes. Thus, if most of the AuNPs are internalized by CLME, inhibition of the macropinocytosis could not provide significant spectral changes in the SERS spectra.

Even CytoD inhibits macropinocytosis by blocking actin polymerization and Aml interfere with Na^+/H^+ exchangers, same changes were observed because at the end both of them block macropinocytosis and development of macropinocytosis pathway is interrupted and changes in the SERS spectra were obtained by the loss of macropinocytosis. On the other hand, when the effects of the inhibitors were compared, it can be concluded that Aml caused more dramatic changes than CytoD. It could be caused by two separate reasons. First of all, mechanism of action of Aml is completely differ from the CytoD and inhibition of Na^+/H^+ exchanger could cause more massive disruptions in not only macropinocytosis process such as impaired acidification, signaling, cytoskeleton remodeling [423,424] but also cellular functions such as protein synthesis [446,447], tyrosine kinase activity [448] and the Na^+/K^+ -ATPase [449,450]. As a second reason, Aml caused AuNPs uptake decrease up to 19 percent while CytoD caused uptake decrease up to 82 percent at the highest concentration. Thus, the amount of AuNPs internalized after Aml treatment is lower than the CytoD treatment and this could cause a decrease in the obtained SERS signal and observed as diminish in the peak intensities just like CytoD but more dramatic.

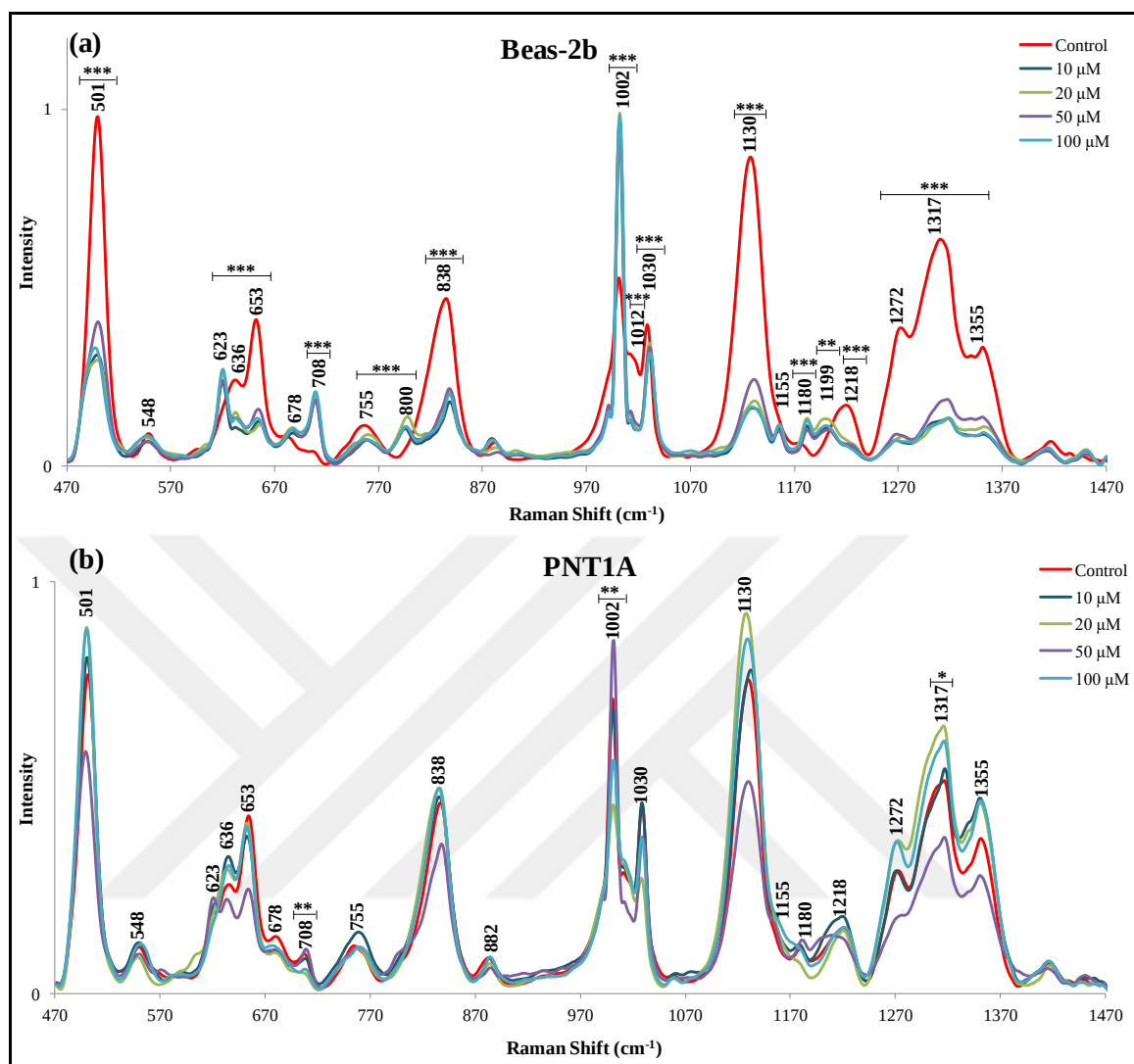


Figure 4.55. Average SERS spectra of (a) Beas-2b and (b) PNT1A cells treated with Aml. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$ with the comparison of 100 μM Aml and control group

4.3.6.4. Statistical Analysis

For the observation of the variation of the SERS spectra, principal component analysis (PCA) and then linear discriminant analysis (LDA) was applied to the obtained average SERS spectra from 30 cells for each treatment group. LDA was applied to the obtained principal component (PC) scores from the PCA analysis. Obtained LDA plots for CytoD and Aml treatment can be seen in Figure 4.56. As seen, for CytoD treatment, better separation was achieved for Beas-2b and also Aml treatment for Beas-2b provide good

separation of the treatment groups from control group. Separation from the control group proves that Aml treatment inhibited macropinocytosis significantly and with the inhibition of one of the endosomal pathways that AuNPs use to internalize, obtained SERS spectra changes significantly. Spectral changes were directly related with the Aml treatment and provided a good separation in the LDA plots.

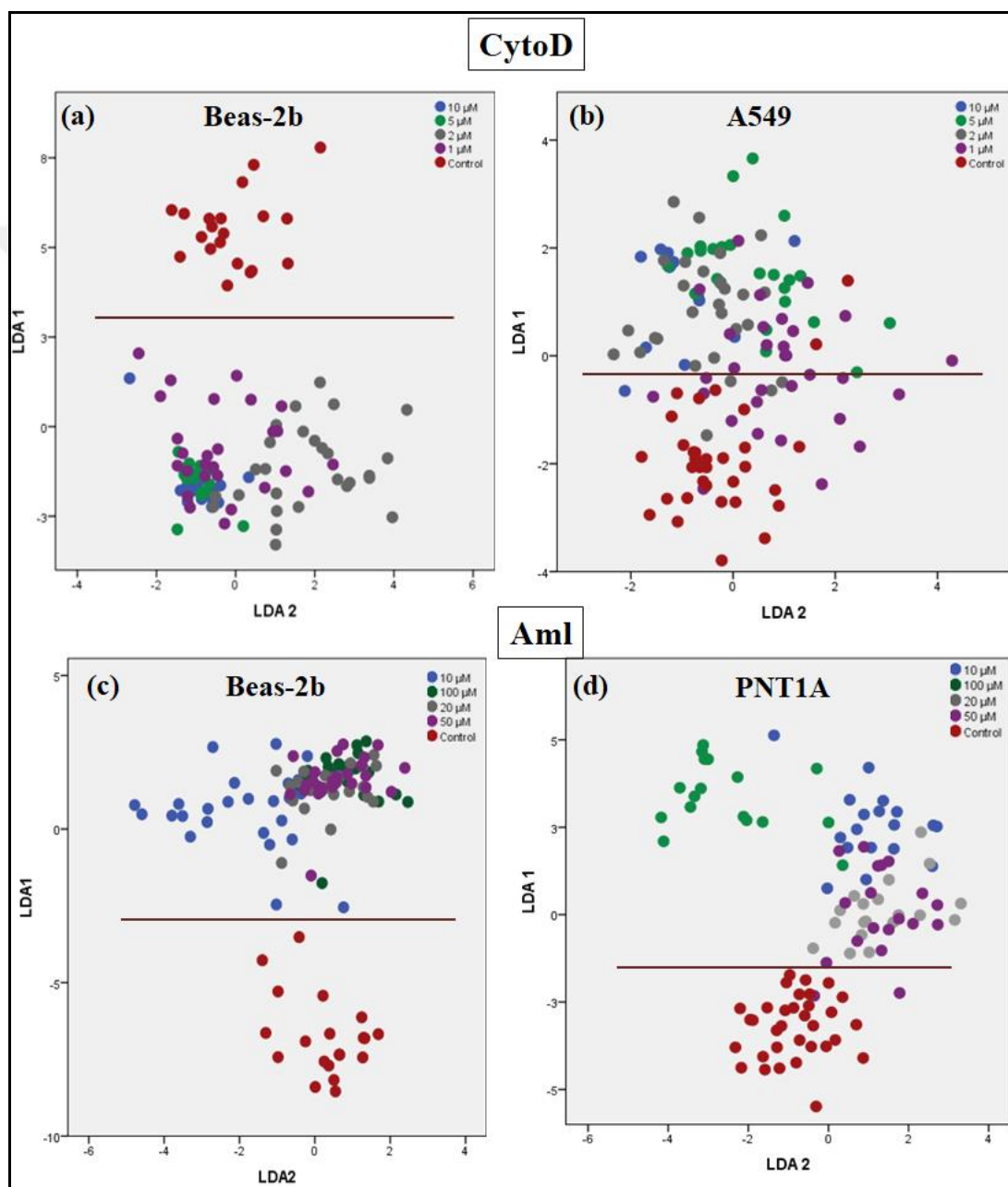


Figure 4.56. LDA Plots of (a) Beas-2b and (b) A549 cells treated CytoD, and (c) Beas-2b, (d) PNT1A cells treated with Aml

4.3.7. Cell Type Dependent Effects on Endocytosis Inhibition

When three different endocytosis pathways were compared based on the obtained SERS data, the spectral changes were found as not only pathway dependent but also cell type dependent. Summary of the cell type dependent uptake of AuNPs could be seen in Table 4.11. For the inhibition of ClME, Scr and Cpm was used. For Beas-2b cell line, Scr inhibited (showed as + in the Table 4.11.) the ClME while Cpm could not (showed as - in the Table 4.8.). Even only one of the inhibitors were found as inhibited the associated endosomal pathway; pathway was considered as related with the AuNPs internalization (showed as $\surd$ in the Table 4.11.) due to the differential effects of each inhibitors. Thus, ClME was found as one endosomal pathway used for the AuNPs uptake for Beas-2b cell line. For A549 cells, same situation was observed for ClME and Scr provided inhibition of ClME while Cpm could not. For HeLa cells, both of the inhibitors did not provide any inhibition which means HeLa cells do not use ClME for the internalization of AuNPs (showed as X in the Table 4.11.). For PNT1A cells, both of the inhibitors inhibited the pathway which shows ClME is one of the internalization pathways of AuNPs for PNT1A cells. Cpm inhibited ClME only for PNT1A cells and this could be explained by the differential mechanisms in the cells. It was shown that PNT1A internalize the least amount of AuNPs when compared with other cell lines as seen in Figure 4.10. Thus, when AuNPs amount was high, affect of Cpm could not be observed in other cell lines while in the PNT1A cells, effect of the Cpm was observed. It should be also noted that PNT1A cells were the most sensitive cell lines against inhibitory treatment due to being a healthy cell line with least uptaken AuNPs rate.

For CaME inhibition, Nys and Fil was used and for Beas-2b and A549 cell lines, both of the inhibitors inhibited the ClME while for HeLa cell line only Nys inhibited the CaME and for PNT1A cell line, only Fil inhibited the CaME. As a consequence, CaME was found as used for the AuNPs internalization in all cell types.

When Dyn was used for the inhibition of DDE, inhibition was achieved for 3 cell lines except PNT1A. If there is an inhibition in either ClME or CaME, an inhibition in the DDE is expected due to being an important protein in both ClME and CaME. However, inhibition in the PNT1A could not observe.

For the inhibition of macropinocytosis, Aml and CytoD were used and for Beas-2b and A549 cell lines, both of the inhibitors provided inhibition of the macropinocytosis. For HeLa cell lines, none of the inhibitor provided any inhibition which means macropinocytosis is not used for the AuNPs uptake in HeLa cells. For PNT1A cells, only Aml inhibited the macropinocytosis.

Consequently, when cell type dependent inhibitions were compared, it can be seen that when cells were selected from the same tissue such as lung for Beas-2b and A549 cells, similar inhibition rates were observed. On the other hand when different tissue based cells were used for the comparison such as HeLa and PNT1A, different inhibitory effect were achieved. On the other hand, when healthy and cancerous cell lines were compared, healthy cell lines responded more to the inhibitors due to having less amount of AuNPs inside the cell and without being resistant to the inhibitory effects.

Table 4.11. Summary of AuNPs internalization pathway choice of cells. √ and X shows the choice of endosomal pathway for each cell line. + and - shows inhibition ability of each inhibitor to the related endosomal pathway. If one of the inhibitors provided inhibition, associated pathway was marked as related

	Beas-2b	A549	HeLa	PNT1A
CIME	√	√	X	√
Sucrose (Scr)	+	+	-	+
Chlorpromazine (CPM)	-	-	-	+
CaME	√	√	√	√
Nystatin (Nys)	+	+	+	-
Filipin (Fil)	+	+	-	+
DDE	√	√	√	X
Dynasore (Dyn)	+	+	+	-
MP	√	√	X	√
Amiloride (Aml)	+	+	-	+
Cytochalasin D (CytoD)	+	+	-	-

For Beas-2b cell line, it was seen that most dramatic changes were observed with CytoD treatment via blocking the actin polymerization in cytoskeleton, and least changes were observed with Scr treatment preventing the dispersion of clathrin lattices. The spectral bands attributed to protein profile changes at 623, 636 and 653 cm^{-1} were not observed at CaME inhibition. The peak intensities at 1199 cm^{-1} and 1218 cm^{-1} attributed to proteins were also found as pathway dependent. The intensity of the band at 1199 cm^{-1} changed significantly at macropinocytosis and CaME while did not change with ClME. On the other hand, the intensity of the band at 1218 cm^{-1} changed significantly at macropinocytosis and ClME while did not change at CaME. Moreover, the intensity of the bands at 755, 882, 1355 cm^{-1} attributed to proteins was changed only with macropinocytosis.

For A549 cell line, again CytoD treatment caused most dramatic changes in the SERS spectra. The peak intensity changes at 636 cm^{-1} (protein), 755 cm^{-1} (Trp symmetric breathing), 800 cm^{-1} (Phosphate ion interactions), and 1155 cm^{-1} (protein C-C and C-N stretching) were found as macropinocytosis dependent while at 548 cm^{-1} (attributed to cholesterol) was found as CaME dependent and 1030 cm^{-1} (Phenylalanine, C-N stretching) as ClME dependent.

For PNT1A cell line, not only Scr but also Fil treatments caused significant changes in the SERS spectra. It can be seen that the band at 800 cm^{-1} (attributed to phosphate ion), 1155 cm^{-1} (protein C-C and C-N stretching vibrations), and 1199 cm^{-1} (Trp ring breathing) are ClME dependent changes, and the bands at 882 cm^{-1} and 1180 cm^{-1} originating from proteins are CaME dependent changes. As a result, the intensities of the peaks attributed to proteins were found to significantly vary in each pathway in a pathway dependent manner.

For HeLa cell line, fewer changes were observed at SERS spectra. Only Nys treated and Dyn treated SERS spectra were obtained because HeLa cells were found as use only CaME for the uptake of AuNPs. When obtained SERS spectra were compared, not significant changes were achieved with Nys treated HeLa cells while Dyn treatment provided significant intensity changes in the peaks originating from endosomes (501 and 1130 cm^{-1}) and proteins (623, 708, 882 cm^{-1}). Consequently, it can be said that pathway dependent changes is also observed for HeLa cells even with slight changes. With the use of intensity changes in the specific bands, endosomal pathway choice of AuNPs can be interpreted.

4.3.8. Inhibition of Receptor Mediated Endocytosis

4.3.8.1. Cytotoxicity of NaN_3 and dG

Cytotoxicity of the receptor mediated endocytosis inhibitors NaN_3 and dG were assessed with WST-8 cell proliferation assay as shown in Figure 4.57. As seen, NaN_3 caused cytotoxic effect on all cell lines with cell viability reduction up to 34 percent for Beas-2b, 35 percent for A549, 24 percent for HeLa and 34 percent for PNT1A while dG caused cell viability decrease only in Beas-2b cell line up to 85 percent.

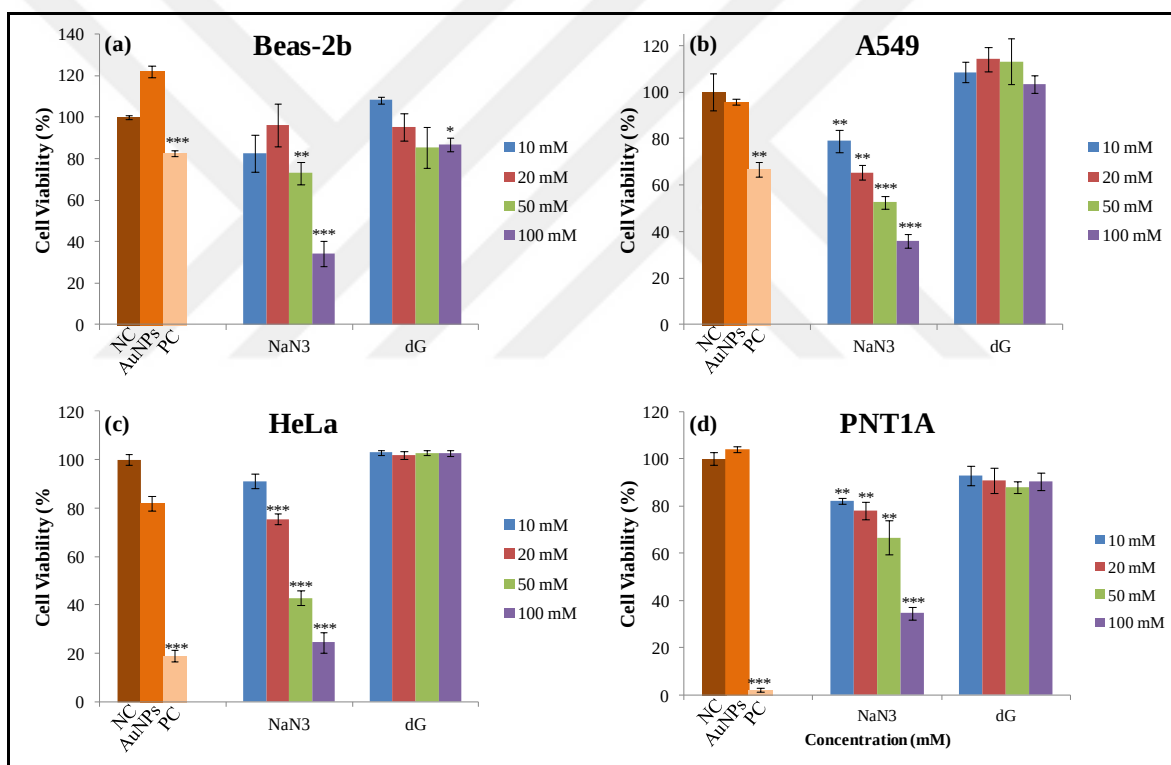


Figure 4.57. WST-8 Cell Proliferation results of (a) Beas-2b, (b) A549, (c) HeLa, and (d) PNT1A after 2 h exposure to NaN_3 and dG. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$. PC: Positive control (10 percent DMSO was used as positive control), NC: Negative control

4.3.8.2. Uptake Inhibition by NaN_3 and dG

Receptor mediated endocytosis was inhibited with the use of NaN_3 and dG and obtained results were shown as SSC shift decrease from flow cytometry as seen in Figure 4.58. As shown, both inhibitors caused decrease in the uptake of AuNPs in all cell lines with differential decrease rates via the ATP depletion effect. With NaN_3 treatment, AuNPs internalization was reduced up to 64 percent for Beas-2b cells, 23 percent for A549 cells, 72 percent for HeLa cells and 57 percent for PNT1A cells. Highest decrease was achieved for A549 cell line with NaN_3 treatment. Moreover, highest decrease of the AuNPs internalization was achieved by the dG treatment of HeLa cells and it decreased internalization up to 24 percent. dG also caused AuNPs internalization decrease for PNT1A cells up to 58 percent, 71 percent for Beas-2b cells, and 85 percent for A549 cells. Even both of the inhibitors were used as ATP depletion agents; they caused different uptake decreases due to their mechanisms of action. NaN_3 depletes ATP by inhibiting oxidative phosphorylation [416] and it could also affects K_{ATP} channels, cellular morphology and can induce apoptosis [451-455]. Besides, dG depletes ATP by inhibiting glycolysis as a glucose analog [417,456,457] and it could also interfere with cellular metabolisms such as N-linked glycosylation, mitochondrial reactive oxygen species (mROS) production, autophagy and ER stress [418,458]. Thus, they can provide different responses with interfering different cellular metabolisms.

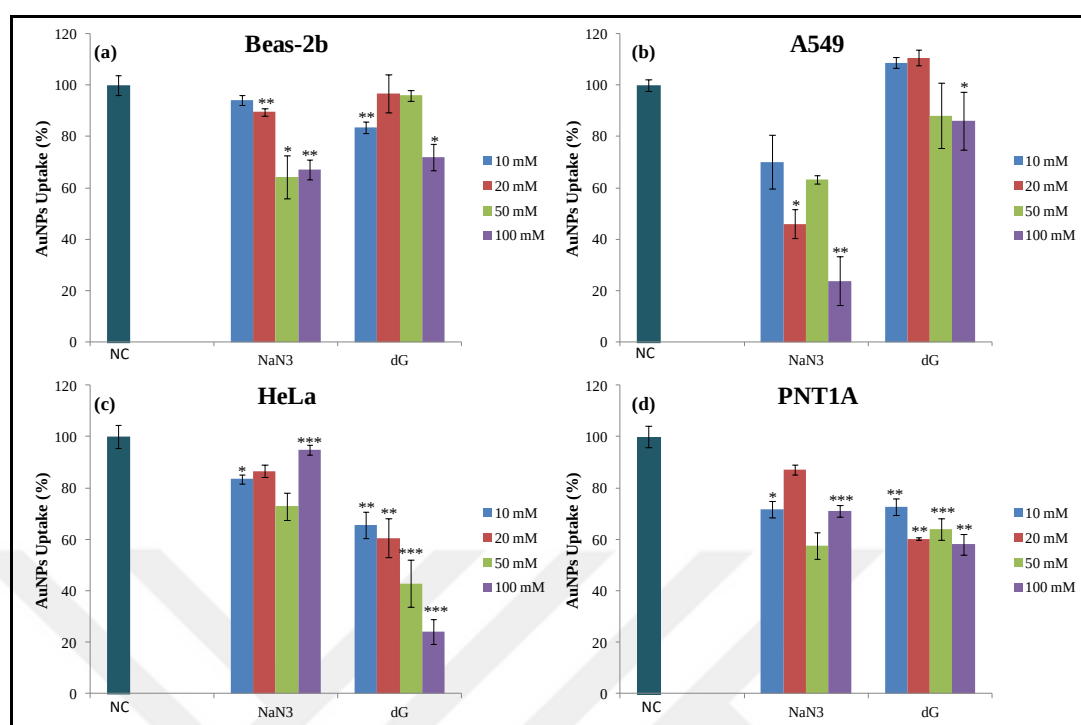


Figure 4.58. Flow cytometry analysis of a) Beas-2b, b) A549, c) HeLa, and d) PNT1A after 2 h exposure to NaN₃ and dG then 22 h AuNPs. NC: Negative control. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$

4.3.8.3. Apoptosis/Necrosis Assay

In addition to the cell proliferation assay, Apoptosis/Necrosis assay was also used for the investigation of the effect of the ATP depletion agents on the cell death mechanisms due to their high cytotoxic effects. Figure 4.59. shows Apoptosis/Necrosis assay results with whole cell population (Figure 4.59.a, c, e and g) and subtracted cell death mechanisms induction results were also shown (Figure 4.59.b, d, and f) which is obtained when live cell population percent was removed from the results to see clear induction of apoptosis and/or necrosis. Obtained results showed that, similar to cell proliferation assay results, NaN₃ caused apoptosis and/or necrosis on the cell populations while dG did not induce any significant cell death mechanism when compared with control groups. For Beas-2b cell line, NaN₃ caused significant necrosis up to 3.6 percent of the population from 0.2 percent and for A549 cell line, NaN₃ caused significant late apoptosis up to 1.4 percent from 0.7 percent and for HeLa cell line, early and late apoptosis was induced up to 5.3 percent from

0.8 and 4.2 percent from 0.7 percent, respectively with NaN_3 treatment. For PNT1A cell line, late apoptosis was induced up to 3.4 percent of the population from 0.7 by NaN_3 .

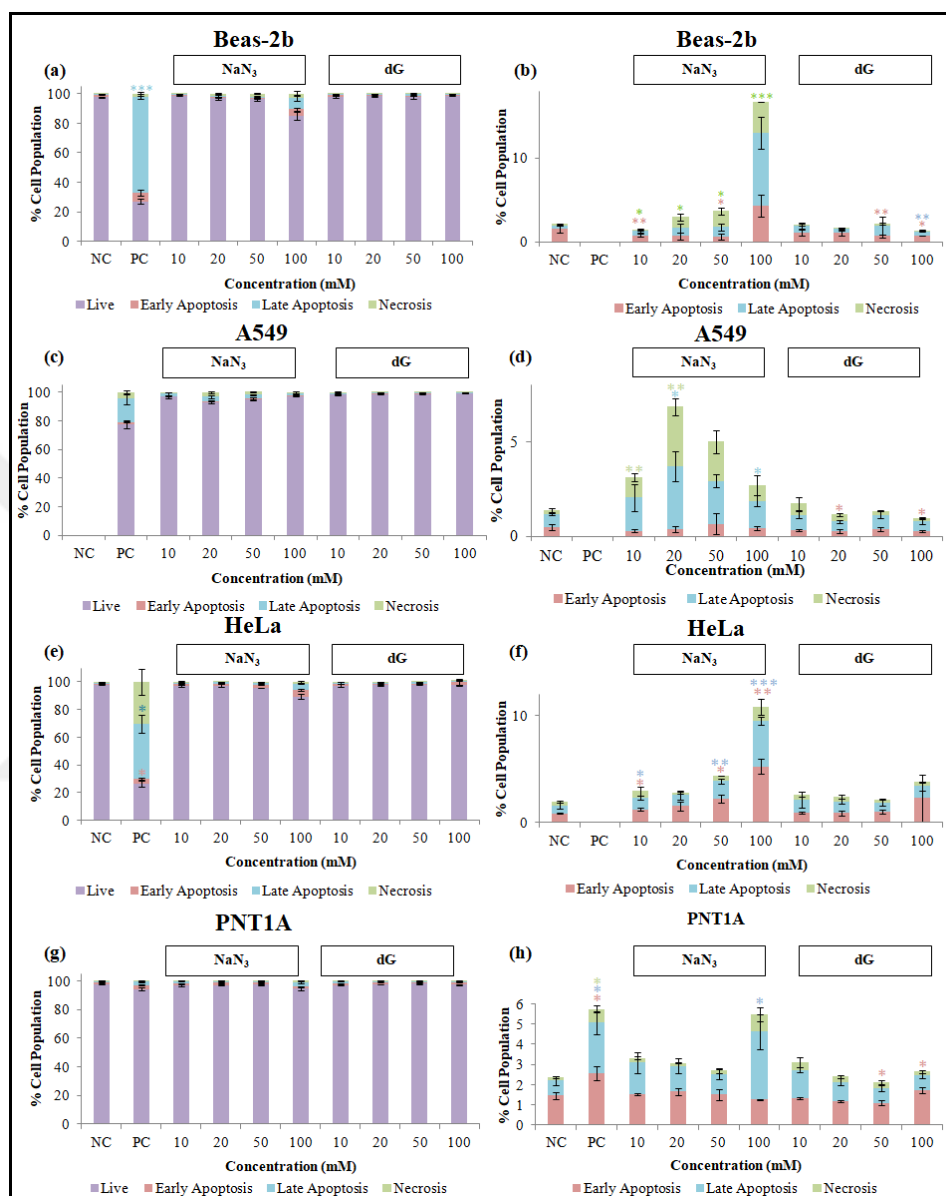


Figure 4.59. Apoptosis/necrosis assay results of a, b) Beas-2b, c, d) A549, e, f) HeLa, and g, h) PNT1A after 2 h exposure to NaN_3 and dG then 22 h AuNPs. (a), (c), (e), (g) shows whole cell population with live, early and late apoptosis and necrosis percentages and (b), (d), (f), (h) shows only early and late apoptosis and necrosis which is obtained with the elimination of live population from the results. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$. PC: Positive control (10 percent DMSO was used as positive control), NC: Negative control

4.3.8.4. ATP Assay

For the ATP depletion, ATP amount was investigated by ATP Assay with NaN_3 and dG treatment as shown in Figure 4.60. It was found that, both NaN_3 and dG caused significant ATP reduction in Beas-2b cells up to 5 μM and 0.9 μM for NaN_3 and dG respectively from total 17 μM ATP. For A549 cell line, ATP amount was decreased by NaN_3 up to 5 μM and by dG up to 24 μM from total 38 μM . For HeLa cell line, NaN_3 caused ATP amount decrease up to 17 μM from 60 μM and no ATP decrease was found for dG treatment. For PNT1A cell line, NaN_3 decreased ATP amount up to 13 μM from 20 μM and dG cause ATP amount decrease up to 3 μM with 20 mM treatment and no ATP reduction was observed with highest dose.

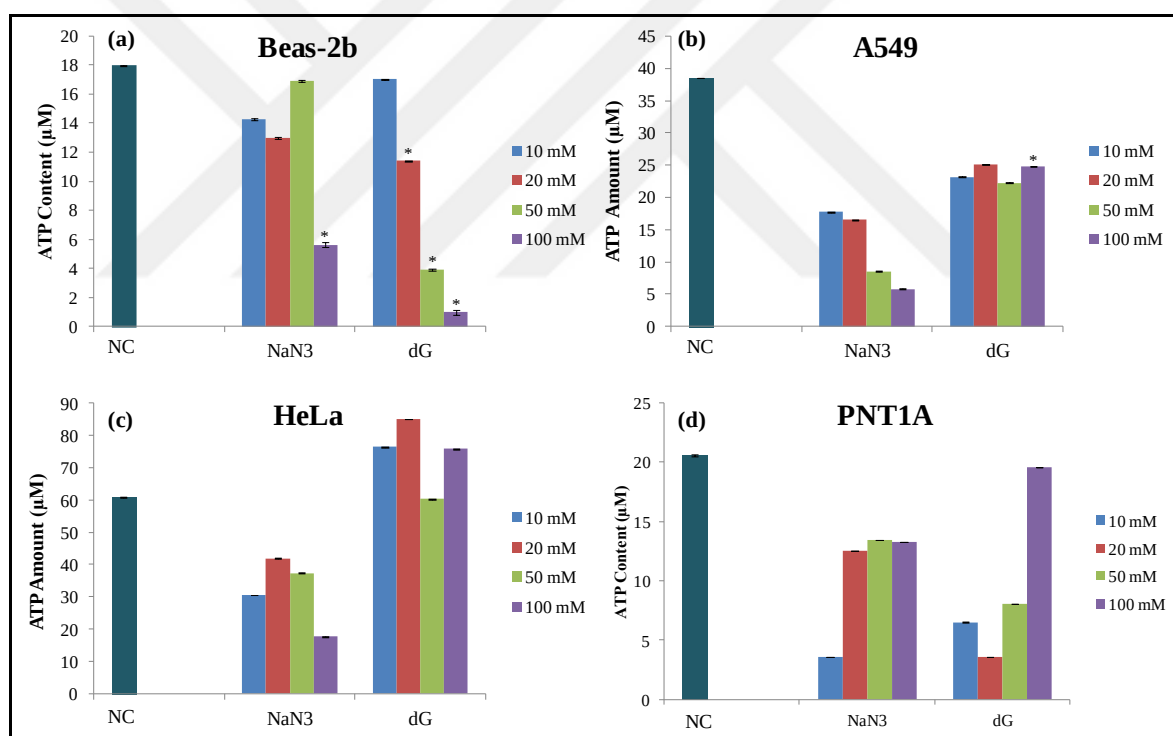


Figure 4.60. ATP assay results of a) Beas-2b, b) A549, c) HeLa, and d) PNT1A after 2 h exposure to NaN_3 and dG then 22 h AuNPs. NC: Negative control. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$

4.3.8.5. SERS Measurements

Receptor mediated endocytosis was inhibited with NaN_3 and dG and obtained SERS spectra belong to the 4 cell lines treated with NaN_3 and dG are shown in Figure 4.61. and Figure 4.62., respectively.

It is known that NaN_3 cause ATP depletion by inhibition of oxidative phosphorylation which is caused by the inhibition of cytochrome oxidase, the mitochondrial transport chain enzyme [416]. Moreover, NaN_3 also affects K_{ATP} channels, cellular morphology and can induce apoptosis [451-455]. On the other hand, dG depletes ATP by inhibiting glycolysis as a glucose analog. It is internalized into the cells like glucose and interferes with glucose metabolism as inhibition of hexokinases and phosphoglucose isomerase [417,456, 457]. As a result, glycolysis and oxidative phosphorylation are disrupted, ATP production levels is decreased [419,459]. It can also interfere with N-linked glycosylation, induce mitochondrial reactive oxygen species (ROS), autophagy and ER stress [418,458].

Moreover, endocytosis requires energy for the movement of the formed vesicles, acidification of vesicles, fusion of vesicles, and coordination of these events to achieve internalization of particles [460,461]. Thus, when AuNPs were internalized by cells in the presence of NaN_3 or dG, surrounding environment would be changed and this is reflected in the SERS spectra.

When NaN_3 and dG are used for ATP depletion, endocytosis is affected in different steps. In the early steps of endocytosis, ATP is used for the coated pit assembly and vesicle budding [460,461]. When coated pit assembly and vesicle budding is blocked, vesicle cannot be scissored from the membrane and coated vesicle cannot be formed, cannot mature to late endosomes and merge with the lysosomes to form endolysosomes. When this dynamic structure was interrupted with an agent, dynamic surrounding environment of the internalized AuNPs will be change and be reflected on the obtained SERS spectra. When obtained SERS spectra were considered, the peak intensities at 501 cm^{-1} (S-S), 1130 cm^{-1} (Phospholipid) were found to be decreased significantly in all SERS spectra regardless of cell type. It is known that formation or deformation of the endosomes based on cysteine rich proteins can be interpreted from the peak intensity at 501 cm^{-1} [388] and the intensity decrease at 1130 cm^{-1} originating from phospholipids shows decrease in the formed endosomes [462]. Furthermore, in the late steps of the endocytosis, early

endosomes mature and late endosomes are formed with exchange of membrane components, change in the fusion partners, formation of additional intraluminal vesicles (ILVs), drop in the luminal pH, acquisition of lysosomal components and change in morphology and movement to the perinuclear area [2,463]. Luminal pH is regulated by vacuolar ATPases (V-ATPase) concentration in the membrane, selection of V-ATPase isoforms and association and dissociation of the subunits of V-ATPases. Subunits of V-ATPase are V₀, which serve as a transmembrane pore for protons, and V₁, which binds and hydrolyze the ATP as a soluble part [62,95]. V-ATPases are responsible for the accumulation of H⁺ inside the lumen of the endocytic vesicle in an ATP-dependent manner [95,464]. Intravesicular acidification obtained by V-ATPases is required for the recruitment of proteins required for the maturation of endocytic vesicle such as Arf1, GTPases and coat protein complex (COP) [465,466]. On the other hand, regulatory event for the maturation of early endosomes to late endosomes such as Rab5/Rab7 conversion and PI conversion cannot be achieved which required many proteins involvement, exchange in the membrane components, change in the fusion partners and drop in the pH and this situation can be observed as changes in the peak intensity of proteins corresponding peaks at the SERS spectra. As seen in Figure 4.61. and Figure 4.62., the peak intensities at 708 cm⁻¹ (Met), 1002 cm⁻¹ (Phe) were found to be increased significantly in all SERS spectra which could be caused by disassemble and accumulation of proteins responsible of early steps of the endosomes such as coated pit formation and vesicle budding. When endosomal maturation is blocked, recruited proteins could not be recycled back and accumulate in the endosomal region. On the other hand, the peak intensities at 1012 cm⁻¹ (Trp), 1030 cm⁻¹ (Phe), 1271 cm⁻¹ (Protein (α -helix)), 1317 cm⁻¹ (Amide III, α -helix), 1355 cm⁻¹ (Protein, β -sheet) were found to be decreased in all SERS spectra again regardless of cell and treatment type. This can be caused by the recruitment fail of the proteins to the endosomal region required for the maturation of early endosomes to late endosomes and endolysosomes.

Acidification by V-ATPase provides uniquely acidic lysosomal pH of ~4.5 which is essential for the enzyme function. Acidic environment also provides conformational changes for the inactive enzymes [467]. Furthermore, protease activity in an acid dependent manner is also crucial for processing maturation of precursor proteins [464]. If conformational changes and autoactivation process of proteases cannot achieve in the late

endosomes, protein profile will be changed when compared with the control groups and resulted in the changed protein corresponding peaks as disappear at 636 cm^{-1} (Protein), 653 cm^{-1} (Tyr) and 1218 cm^{-1} (Protein) and arise as new at 623 cm^{-1} (Protein), 1155 cm^{-1} (Protein), 1180 cm^{-1} (Tyr, Phe), and 1199 cm^{-1} (Trp). Moreover, ATP depletion can also affect the F-actin assembly and cause unregulated actin polymerization [468, 469] which are the fundamental processes for the development of endocytosis including membrane invagination, vesicle formation and movement of endosomes [171, 188, 470]. In all steps of the endocytosis, different proteins are required and they are recruited to the endosomal region and after they used for the required process (in this case including actin nucleation, filament formation, membrane invagination and vesicle formation with budding), they are disassemble from the vesicle and some new regulatory proteins are recruited for later steps. With the unregulated actin polymerization, this process could be affected and this can be seen as protein profile change in the SERS spectra as in the changes at two spectral regions as $623/636\text{--}653\text{ cm}^{-1}$ and $1218/1155, 1180, 1199\text{ cm}^{-1}$ [462].

dG and NaN_3 do not only interfere with endosomal pathway by ATP depletion, they also interrupt other pathways including cellular death mechanisms, actin polymerization, mitochondrial activity and glycolysis [416,468,471,472]. It should be also noted that, remaining V-ATPases are also localized in the plasma membrane used for the H^+ balance and secretory vesicles used for physiological homeostasis regulation by the activity of secreted hormones [464,473]. Effects on the cellular processes can also be observed on the SERS spectra when AuNPs able to escape from the endocytic vesicles and accumulated in the cytosol as individual NPs or aggregates besides the effects on the endocytosis [353].

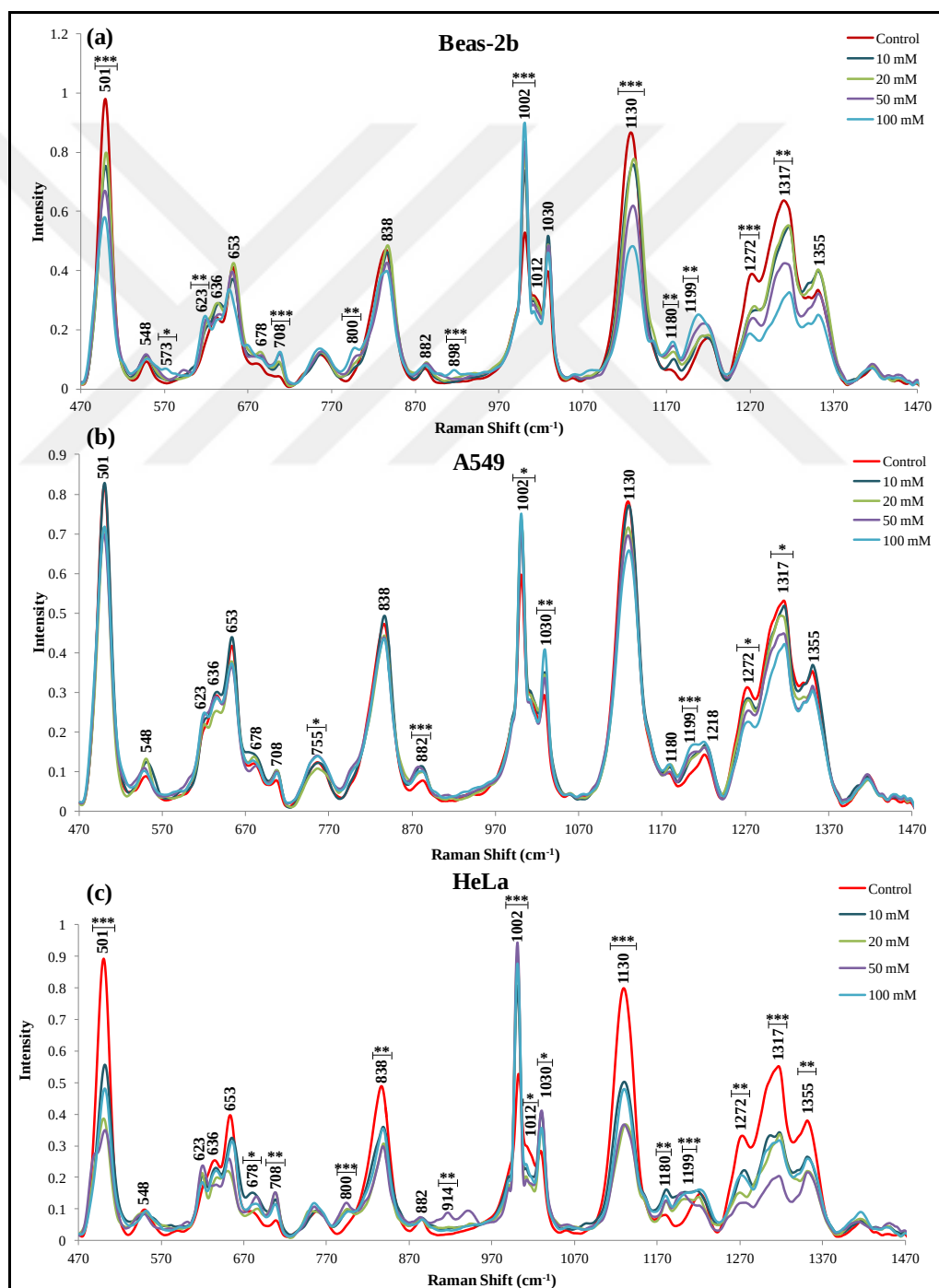
When cells were treated with NaN_3 , it inhibits mitochondrial ATP production as being electron transport inhibitor. Furthermore, it reduces mitochondrial activity and increases mitochondrial ROS which are resulted in the mitochondria mediated apoptosis [416]. NaN_3 caused apoptosis can be seen in Figure 4.59. It also causes morphological changes in the cells such as cell nuclei shrinkage and increased chromatin condensation [416]. That much alteration in the cellular mechanisms caused changes in the SERS spectra. For instance, peak intensities originating from proteins (573 cm^{-1} , 708 cm^{-1} and 1002 cm^{-1}) were found to change significantly with NaN_3 treatment and peaks at 914 cm^{-1} (Ribose) and 814 cm^{-1} (Adenine) were changed only for NaN_3 which could be resulted in the activation of apoptosis. It is known that NaN_3 caused upregulation the expression levels of Bax and

cytochrome c and downregulation the expression levels of Bcl-2 and procaspase [416] which are all related with the apoptosis [451,452,474,475]. With the activation of apoptosis, caspases were activated which are used for the cleavage of the different substances in cytoplasm and nucleus including DNA, cytoskeletal proteins. With morphological changes and alterations in the protein profile as well as nuclear structure, different spectral bands were observed attributed to proteins and nuclear structures such as ribose and adenine [476].

Besides the general changes in the SERS spectra with NaN_3 treatment, cell type dependent changes were also observed as seen in Figure 4.61. When cellular SERS spectra were compared, it can be seen that most effected cell line is HeLa with highest decrease in the peak intensities and the least effected cell line is PNT1A with very few significant peak intensity changes. When changes in the peak intensities were compared, peak intensities corresponding to ribose (914 cm^{-1}) and proteins (1012 cm^{-1} and 1030 cm^{-1}) were found to change only for HeLa and peak intensities corresponding to nucleotides (573 cm^{-1} and 898 cm^{-1}) changed only for Beas-2b whereas peak intensities corresponding to phosphate ion interactions (800 cm^{-1}) and proteins (1199 cm^{-1} and 1355 cm^{-1}) changed only for PNT1A. This could be related with the different effects of NaN_3 on the cell lines. Although NaN_3 treatment caused cytotoxicity (Figure 4.57.) and ATP decrease (Figure 4.60.) in all cell lines, it induced apoptosis significantly in both Beas-2b and HeLa cells whereas increase in the apoptosis rate of PNT1A cells was not significant when compared for the highest concentration of NaN_3 as in the Figure 4.59. And cellular changes can be caused by the induction of different cellular death mechanism in Beas-2b as necrosis and HeLa as early and late apoptosis.

With the activation of necrosis, cell integrity is lot and organelles are disintegrated which cause release of the cellular content and contact with the internalized AuNPs [316]. This situation can be observed in the SERS spectra as unique peaks appearance when only necrosis is activated by treatments as seen in NaN_3 treated Beas-2b cells at all doses and NaN_3 treated A549 cells at low doses. As seen in the SERS spectra, peak at 573 cm^{-1} originating from cytosine, guanine, and 898 cm^{-1} originating from adenine is appeared only when Beas-2b cells were treated with NaN_3 at high doses which cause significant necrosis in cells. Furthermore, it is known that appearance of the peaks originating from the nucleotides is known as a result of DNA fragmentation during cell death [355], thus cell

type dependent effect of the NaN_3 caused differential SERS spectral changes for each of the cell line. Even significant necrosis was observed at A549 cells for low dose treatment of NaN_3 , dramatic changes were not observed at SERS spectra. This differential effect was probably caused by the dramatic effect of NaN_3 on healthy cell line Beas-2b where almost 20 percent of the cell population was found at apoptosis and necrosis whereas not much effect on cancerous cell line was observed with NaN_3 treatment where only 5 percent of the cell population was found in apoptosis and necrosis state.



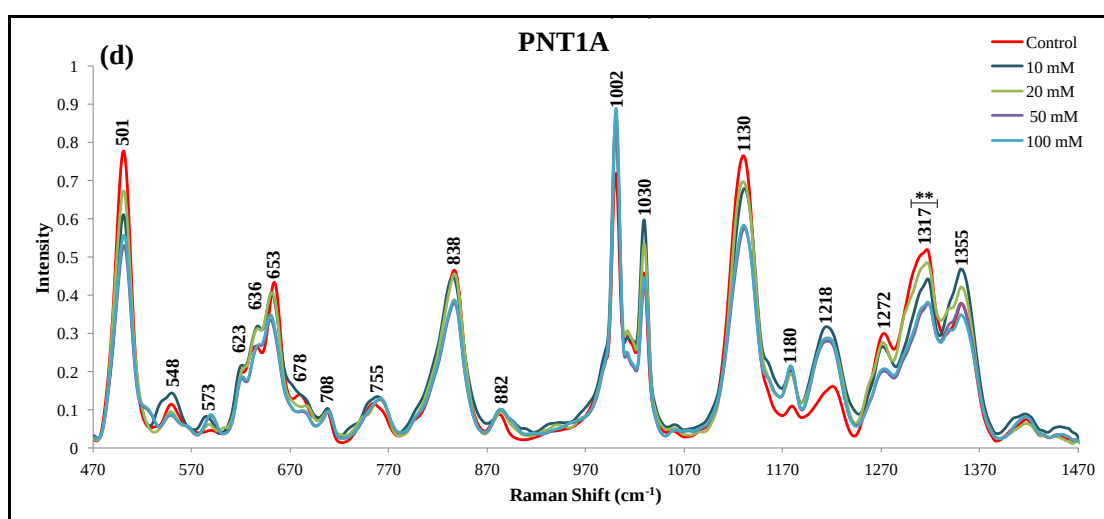


Figure 4.61. Average SERS spectra of (a) Beas-2b, (b) A549, (c) HeLa, and (d) PNT1A cells treated with NaN_3 . Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$ with the comparison of 100 mM NaN_3 and control group

When cells were treated with dG, it can deplete energy but also inhibit glycolysis, induce mitochondrial ROS, interfere with N-linked glycosylation and induce autophagy [418,419]. When compared with NaN_3 treatment, more dramatic changes were observed at SERS spectra as seen in Figure 4.62. for all 4 cell lines with dG treatment. The peak intensities originating from proteins at 755, 838, 882, and 1155 cm^{-1} were found to be changed significantly only for dG treatment. Besides, only peak intensity at 1155 cm^{-1} was found to increase while the rest is decreased. This could be related with the disrupted recruitment and disassemble of required proteins for different metabolisms in the cells besides the endocytosis which lead to change in the protein profile in the whole cell with dG treatment.

As an important cellular metabolism, dG can inhibit glycolysis by being glucose analog. This inhibition process starts with the internalization of dG by glucose transporters (GLUT) like glucose. Then, dG is phosphorylated by hexokinases (HK) to form 2-deoxy-D-glucose-6-phosphate (dG-6-P). Formed dG-6-P cannot be metabolized via glycolysis and accumulate in the cell. It inhibits HK noncompetitively and phosphoglucose isomerase (PGI) competitively [417,456,457]. With the inhibition of first steps of glucose metabolism, glycolysis and oxidative phosphorylation are disrupted, ATP production level is decreased and cell growth is inhibited [419,459]. On the other hand, inhibition of

glycolysis lead autophagy with the activation of AMP-activated protein kinases (AMPK) which is the result of the decreased levels of ATP and increased levels in the AMP/ATP ratio and ER stress. In ER, dG cause increased Ca^{2+} efflux which induce elevated cytoplasmic Ca^{2+} concentrations [477]. This situation activates Ca^{2+} /calmodulin-dependent protein kinase β (CaMKK β) and its downstream target AMPK which is resulted as autophagy induction [478]. Another marker of autophagy Beclin1 is also activated by dG which disengages Beclin 1 from Bcl-2 which act as a key anti-apoptotic regulatory protein of the mitochondrial death pathway and also negatively regulate Beclin 1[458].

dG interferes not only glycolysis but also N-linked glycolysation with the resemblance with mannose. It is converted to dG-GDP before competing with mannose-GDP during the initial steps of N-linked glycolysation. Conversion of dG to dG-GDP cause depletion of the chain forming precursor and causes a further disrupted oligosaccharide formation which cause formation of disrupted and unfolded/misfolded glycoproteins which lead to ER stress at the end [417]. Formation of unfolded/misfolded glycoproteins in the ER activates unfolded protein response (UPR) and lead ER stress and death [479,480]. UPR has a defensive function to relieve ER stress with the inhibition of protein translation, reduction of the amount of protein entering ER and increase in the degradation of aberrant proteins [481]. However, with increased ER stress, ER stress specific apoptotic response elements such as C/EBP homologous protein (CHOP) are activated and cause ER stress induced cell death [480,482].

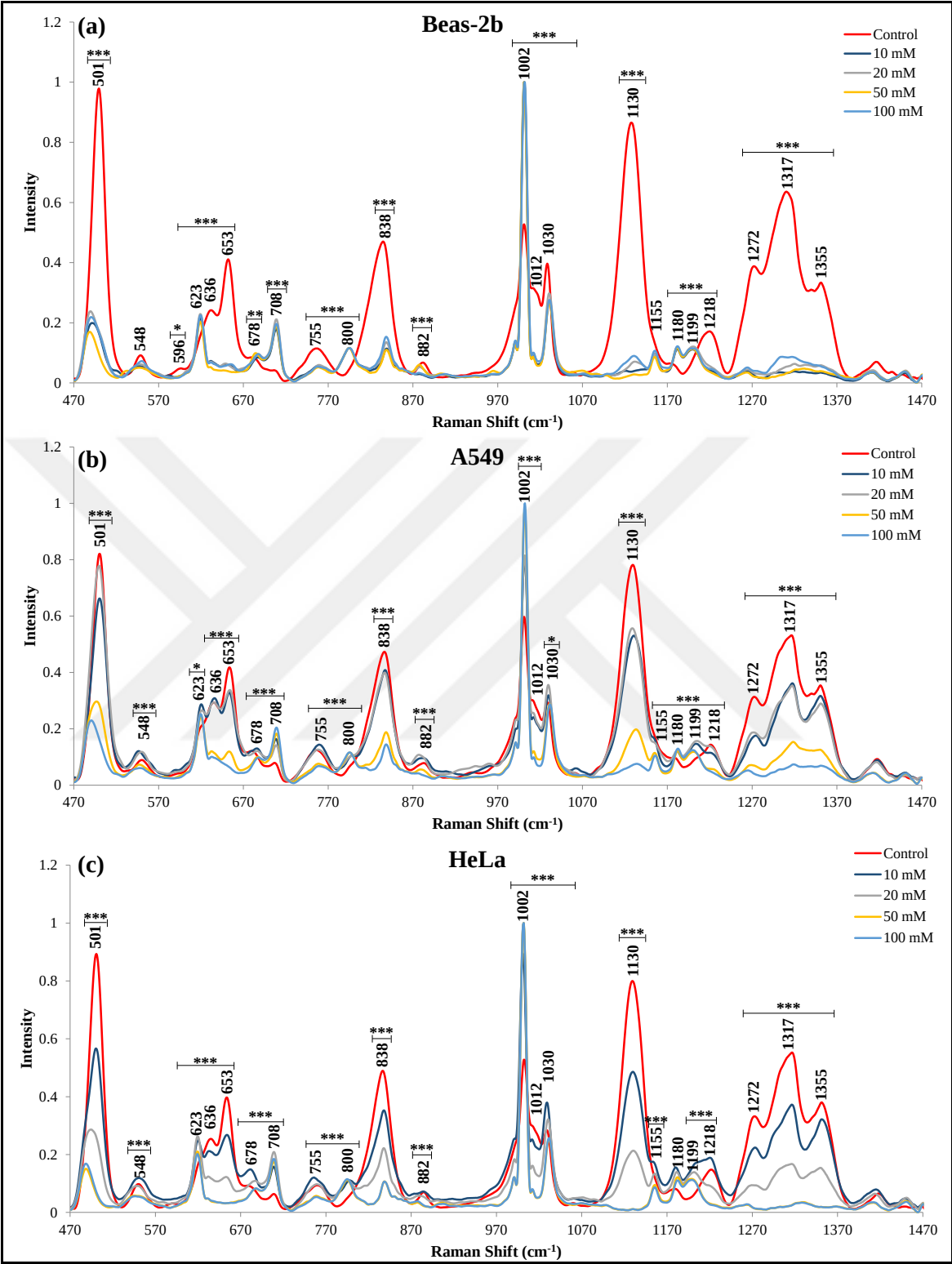
When glycolysis, Ca^{2+} efflux metabolisms were disrupted with dG treatment and ER stress and autophagy were induced, whole cellular protein profile could change which will be observed as peak intensity changes corresponding to proteins (755, 838, 882, and 1155 cm^{-1}).

Furthermore, specific spectral changes were also observed with dG treatment which could not be observed with NaN_3 treatment such as decrease in the peak intensity at 548 cm^{-1} (Cholesterol) and 596 cm^{-1} (phosphatidylinositol). Cholesterol change could be related in two different metabolisms as endocytosis and cholesterol efflux. When apoptosis were induced by ER stress which is induced by dG treatment, caspase activity and cytosolic Ca^{2+} increases and lead cholesterol release and phospholipid degradation from endolysosomal membrane [483]. This can be observed in SERS spectra as changes in the

cholesterol corresponding peaks [367]. As a second mechanism, ATP depletion by dG could cause changes in the cholesterol efflux with the conformational changes in the ATP-binding cassette protein A1 (ABCA1) which is the responsible on the cholesterol homeostasis and HDL metabolism [471] and this change could cause spectral changes as well.

Changes in the phosphatidylinositol (596 cm^{-1}) and also phosphate ion interactions (800 cm^{-1}) could be caused by the altered phosphatidylinositol (PI) metabolism because dG induces Akt phosphorylation independently from altered glucose metabolism and Akt phosphorylation is required for the phosphatidylinositol-3-kinase activity (PI3K) [484]. PI3K is used for the formation of PI3P which is used for the regulation of endosomal maturation. PI3P found on the cytosolic leaflet of EE membranes formed by a PI3K which is VPS34 [90]. VPS34 forms a complex with p150 and Beclin-1, which is also affected by dG directly [458], for the regulation of the VPS34 kinase activity [91]. With the help of formed complex, Rab5/Rab7 switch can be achieved which is among the fundamental process for endosomal maturation [85]. This alteration on the PI and endosomal maturation metabolism could be observed in the SERS spectra as decreased intensity of the peaks corresponding to PI (596 cm^{-1}) and also increased in the phosphate ion interactions (800 cm^{-1}).

When cell-based differences obtained by dG treatment were taken into account, it was found that peak intensity at 573 cm^{-1} originating from cytosine, guanine decreased only for PNT1A, and 596 cm^{-1} originating from phosphatidylinositol decreased only for Beas-2b and 882 cm^{-1} did not change only for PNT1A cells. These cell dependent changes can be caused by the differential effect of dG on each type of cell line. For instance, from the dG treatment, the most effected cell line is Beas-2b and least effected cell line is PNT1A. With dG treatment, uptake rate and ATP amount were decreased in all cell lines. Even uptake rate is decreased most at HeLa cells, Beas-2b cells were more affected with the cytotoxic effect of dG. When cytotoxicity results (Figure 4.57.) were investigated, it can be seen that dG caused cytotoxicity only Beas-2b at highest concentration and apoptosis were induced most at Beas-2b cell line as the induction of both early and late apoptosis when compared with other cell lines. That means even AuNPs internalized by endocytosis and endocytosis is affected by ATP depletion, major cellular metabolism changes such as cell death mechanisms affect cellular SERS spectra than any other effects.



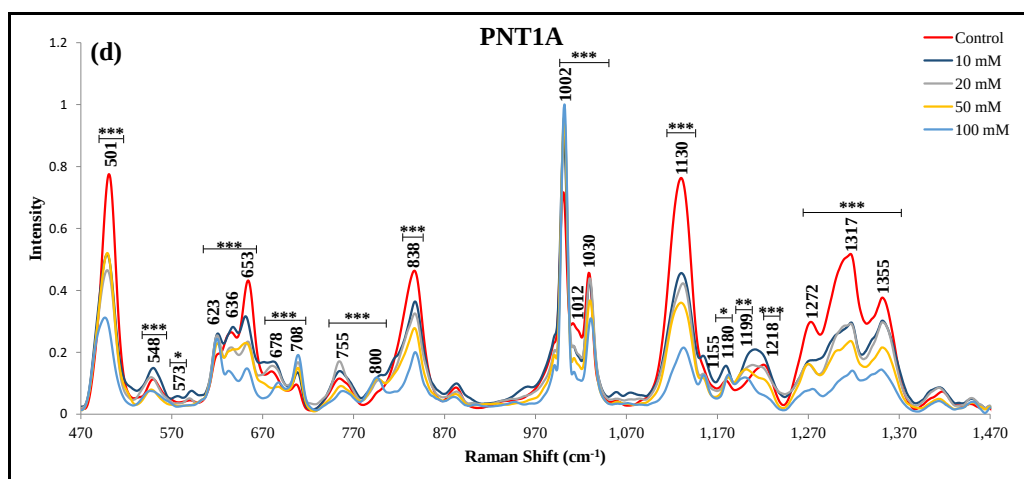


Figure 4.62. Average SERS spectra of (a) Beas-2b, (b) A549, (c) HeLa, and (d) PNT1A cells treated with dG. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$ with the comparison of 100 mM dG and control group

4.3.8.6. Statistical Analysis

For the observation of the variation of the SERS spectra, PCA and LDA was applied to the obtained average cellular SERS spectra after NaN_3 and dG treatment and obtained LDA plots can be seen in Figure 4.63. and Figure 4.64. As seen, for NaN_3 treatment better separation was achieved for HeLa and PNT1A cell lines which are consistent with the changes in the SERS spectra. Most significant changes were observed at HeLa cell line with NaN_3 treatment, and this reflected on the LDA plots. For Beas-2b cell line, not a good separation was achieved even significant changes were observed on SERS spectra which are mainly caused by the scattered dispersion of control group. For dG treatment, best separation was achieved by Beas-2b and HeLa cells where SERS spectra were almost completely changed with increasing concentrations of dG treatment. Although for A549 cell line, SERS spectra was significantly changed by increasing concentrations of dG treatment, control group was overlapped with the lower doses of dG treatment in LDA plots.

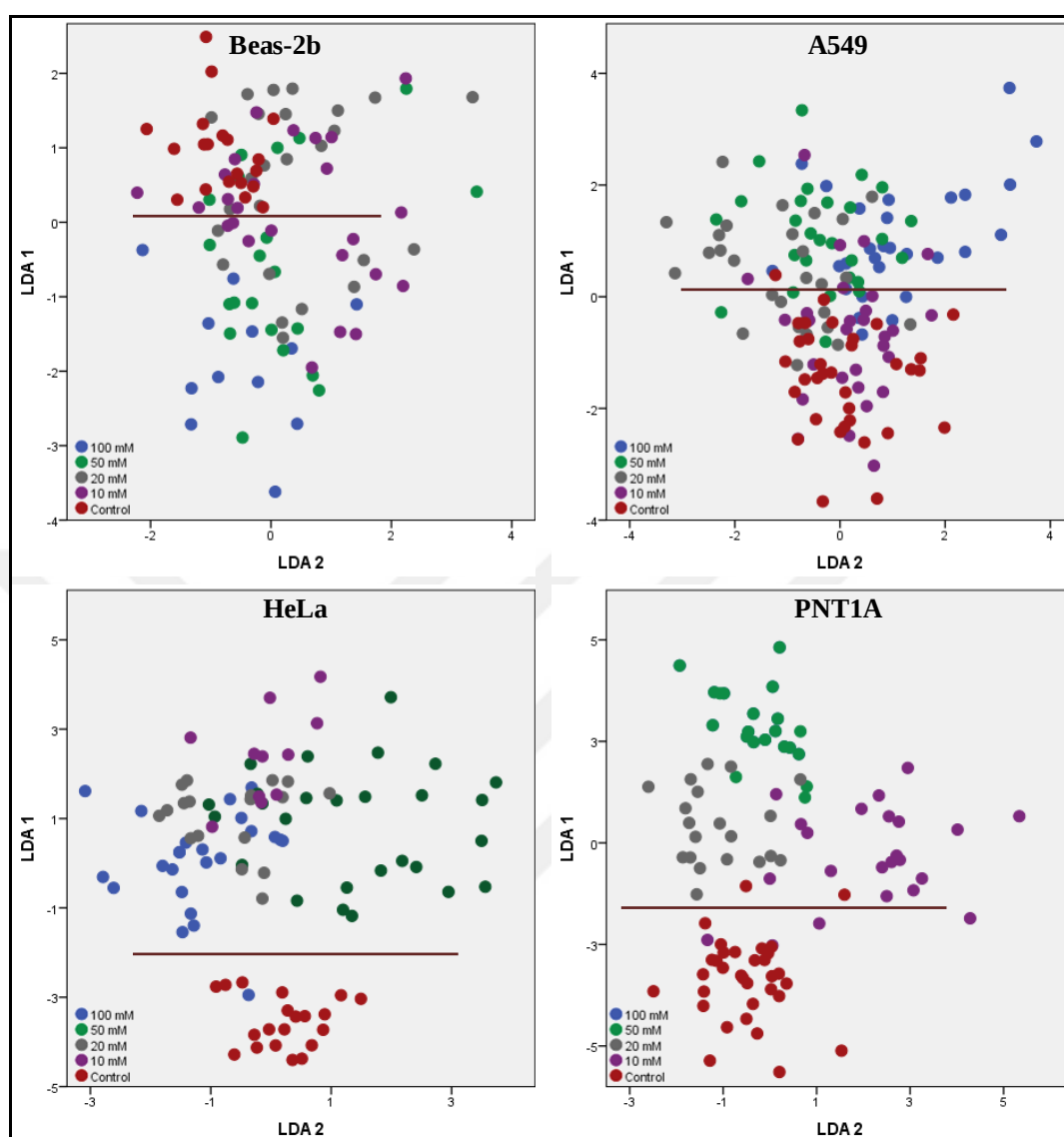


Figure 4.63. LDA Plots of (a) Beas-2b, (b) A549, (c) HeLa, and (d) PNT1A cells treated with NaN_3

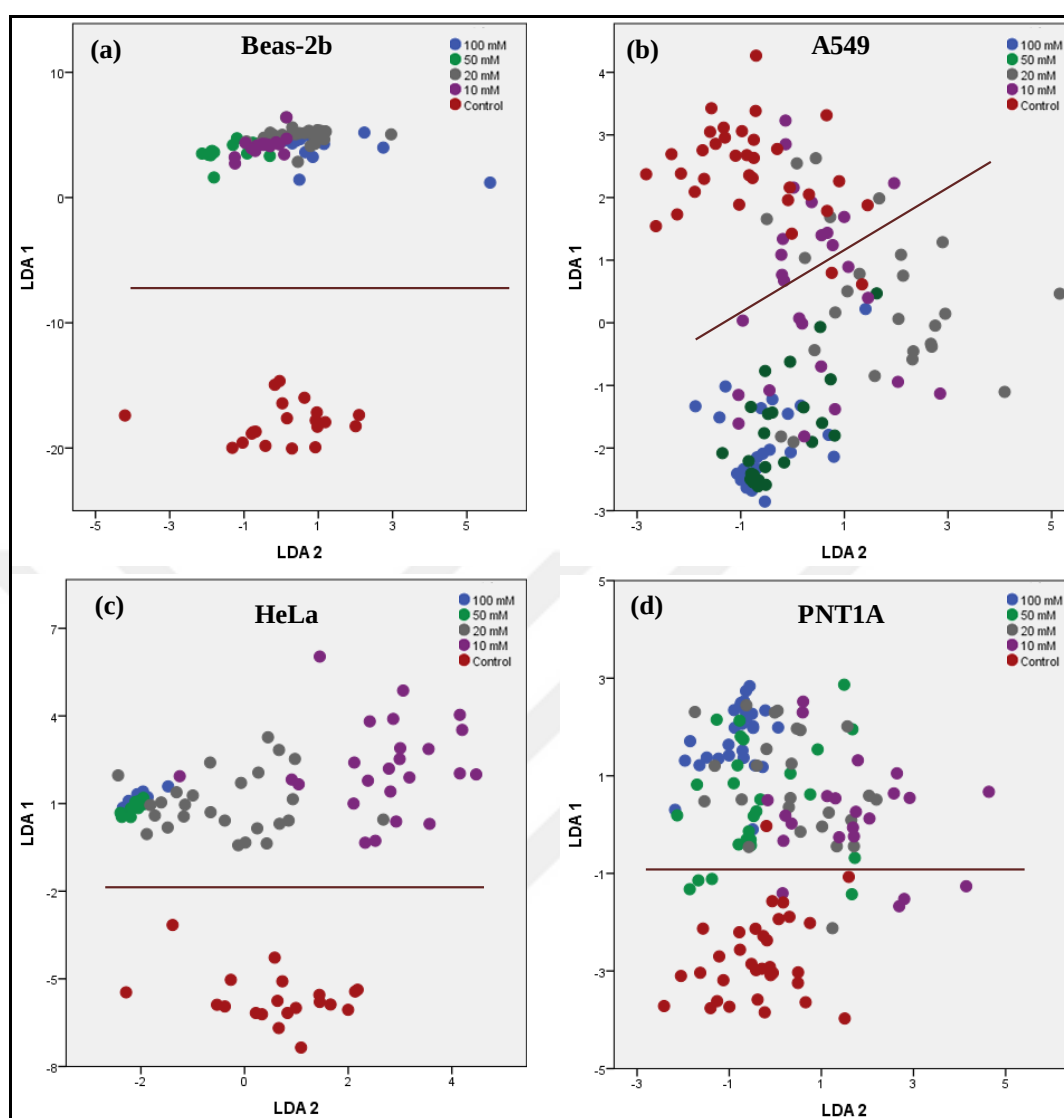


Figure 4.64. LDA Plots of (a) Beas-2b, (b) A549, (c) HeLa, and (d) PNT1A cells treated with dG

Furthermore, p values for the selected Raman shift in SERS spectra were given as Table 4.12.-4.15. For all of the cell lines, when inhibitors were used to inhibit specific pathway, highest concentration provided more significant changes in the SERS spectra, as seen. On the other hand, peak intensity of the same spectral bands did not change in all type of inhibitions. For instance, for A549 cell line 573 cm^{-1} (Trp/cytosine, guanine) and 596 cm^{-1} (phosphatidylinositol) did not change with any type of inhibitor treatment while 573 cm^{-1} changed with NaN_3 treatment for Beas-2b and PNT1A cells and 596 cm^{-1} changed with dG treatment for Beas-2b and NaN_3 treatment for HeLa cell line. Moreover significant changes of the peak intensities show the action mechanism of used inhibitors. As example,

protein profile changes could be observed at 623 cm^{-1} / 626 cm^{-1} , 653 cm^{-1} and 1155 cm^{-1} , 1180 cm^{-1} , 1999 cm^{-1} / 1218 cm^{-1} regions. For Beas-2b cell line, both of the regions were changes significantly with Aml and CytoD treatment for micropinocytosis inhibition while peak intensity changes did not observe with Dyn treatment for receptor mediated endocytosis inhibition. This comparison can be done for also cell type dependent. For HeLa cell line, Aml and CytoD treatment did not provide significant change in the peak intensities of both of the regions while for A549 and Beas-2b cell lines both of the regions were changed significantly. Thus, significance of the peak intensity could help for the interpretation of the SERS spectra based on the action mechanism of the inhibitors as cell type dependent manner.

Table 4.12. Total p values for the SERS spectrum obtained from A549 cells treated with selected inhibitors. Red cells show p value < 0.05, yellow highlight shows p value < 0.01, and green highlight shows p value < 0.001

			Raman Shift (cm ⁻¹)																											
			501	548	573	596	623	636	653	678	687	708	755	800	838	882	898	1002	1012	1030	1063	1130	1155	1180	1199	1218	1272	1317	1352	
A549	CytD	1 μM	0.1339	0.004	-	-	3E-06	0.013	0.1886	0.5761	-	0.0002	0.0323	0.0004	0.8758	-	-	7E-06	0.184	1E-05	-	0.025	0.0272	0.0007	3E-05	0.0308	0.1269	0.0269	0.149	
		2 μM	0.334	0.3259	-	-	0.3234	0.3261	0.3292	0.3262	-	0.3238	0.3264	0.3245	0.3278	-	-	0.3217	0.3269	0.3249	-	0.3291	0.3256	0.3253	0.3259	0.3259	0.3271	0.3278	0.3264	
		5 μM	0.0012	0.6082	-	-	1E-05	0.249	0.0032	0.065	-	8E-08	0.0164	4E-07	0.0195	-	-	1E-09	0.0103	0.0037	-	0.0004	0.7284	6E-05	2E-05	0.2478	0.0004	0.0026	0.1326	
		10 μM	3E-06	0.548	-	-	0.0057	0.0021	1E-07	9E-06	-	5E-06	9E-07	2E-07	4E-08	-	-	5E-11	3E-10	0.8488	-	3E-12	2E-05	0.0007	0.0009	0.0019	3E-10	1E-12	0.0019	
	Sucrose	200 mM	0.3381	0.4967	-	-	0.5923	0.5787	0.0258	0.3961	-	0.7802	-	0.052	0.6983	0.0126	-	0.3151	0.5313	0.0917	-	0.6865	-	0.231	0.007	0.0392	0.3415	0.6864	0.7353	
		300 mM	0.0007	0.2528	-	-	0.2467	0.0521	0.002	0.1519	-	0.0004	-	0.0263	0.0683	0.0035	-	2E-06	0.0425	0.0819	-	0.0005	-	0.0108	3E-06	0.536	0.0032	0.0008	0.2669	
		400 mM	0.0013	0.2601	-	-	0.0836	9E-05	6E-06	0.0892	-	9E-06	-	0.0911	0.0072	0.004	-	1E-07	0.0334	0.0378	-	5E-05	-	0.0033	0.0001	0.2143	0.0018	3E-05	0.0008	
		500 mM	0.0087	0.4948	-	-	0.0621	0.9554	0.0004	0.2223	-	0.0073	-	0.0559	0.0131	0.0035	-	5E-06	0.0318	0.0122	-	0.0024	-	0.0493	5E-05	0.0301	0.0005	0.0008	0.1077	
	Nys	100 nM	0.0216	0.4166	-	-	-	0.216	0.4066	0.8843	-	0.1506	0.1793	-	0.6524	0.5198	-	0.5486	-	0.2565	-	0.9061	-	-	-	0.8232	0.2696	0.1924	0.1948	
		200 nM	0.3076	0.0536	-	-	-	0.5023	0.8672	0.0416	-	0.225	0.1879	-	0.4664	0.9395	-	0.9877	-	0.304	-	0.9987	-	-	-	0.9173	0.8115	0.2637	0.0742	
		300 nM	0.0042	0.1007	-	-	-	0.1797	0.4065	0.317	-	0.0744	0.6453	-	0.3965	0.7275	-	0.5212	-	0.1789	-	0.2328	-	-	-	0.3396	0.7965	0.8669	0.1348	
		400 nM	0.1647	0.0264	-	-	-	0.0421	0.6689	0.5946	-	0.6914	0.0937	-	0.4364	0.0038	-	0.2289	-	0.0828	-	0.4101	-	-	-	0.6051	0.3908	0.702	0.5133	
	Fil	0.1 μM	0.0931	0.2427	-	-	0.0197	0.4736	0.4138	0.8143	-	0.0001	-	-	0.4266	0.1885	-	9E-05	-	1E-05	-	0.1388	-	0.1093	-	0.1534	0.0861	0.0571	0.0662	
		0.5 μM	0.0086	0.1517	-	-	0.0022	0.8215	0.0457	0.8674	-	0.0007	-	-	0.0852	0.0893	-	0.0001	-	2E-05	-	0.0862	-	0.1879	-	0.4414	0.2175	0.0533	0.0779	
		1 μM	0.0947	0.0823	-	-	0.078	0.2244	0.7815	0.3299	-	0.0768	-	-	0.7616	0.0544	-	0.0217	-	0.0016	-	0.8036	-	0.9285	-	0.1356	0.3774	0.9577	0.3798	
		2 μM	0.1387	0.0063	-	-	0.0127	0.2311	0.6049	0.0817	-	0.0163	-	-	0.6405	0.0027	-	0.0005	-	8E-06	-	0.5445	-	0.0158	-	0.0742	0.3767	0.1815	0.2253	
	Dyn	10 nM	0.6933	0.021	-	-	0.0515	0.3117	0.084	0.1747	-	0.0312	-	-	-	-	-	-	-	-	-	0.6137	-	0.4955	-	-	0.0685	0.2002	0.4711	
		20 nM	0.2776	0.5155	-	-	0.0092	0.7573	0.6007	0.7507	-	0.0299	-	-	-	-	-	-	-	-	-	0.0511	-	0.2439	-	-	0.0249	0.0162	0.0628	
		40 nM	0.4663	0.2524	-	-	0.092	0.6388	0.2862	0.7624	-	0.0296	-	-	-	-	-	-	-	-	-	0.3761	-	0.9347	-	-	0.0884	0.0695	0.1446	
		80 nM	0.5937	0.3389	-	-	0.2387	0.3651	0.6604	0.7666	-	0.0411	-	-	-	-	-	-	-	-	-	0.377	-	0.3582	-	-	0.4042	0.5455	0.3367	
	NaN3	10 mM	0.6785	0.0232	-	-	0.1934	0.911	0.4486	0.1601	-	0.2158	0.4298	-	0.3679	0.0014	-	0.0583	-	0.1905	-	0.6632	-	0.1663	0.0256	0.2115	0.293	0.5233	0.9344	
		20 mM	0.2755	0.0707	-	-	0.4779	0.0592	0.3896	0.3689	-	0.2354	0.7727	-	0.5015	0.0146	-	0.0287	-	0.1867	-	0.285	-	0.0602	0.0219	0.3838	0.3171	0.3353	0.0782	
		50 mM	0.197	0.0684	-	-	0.0291	0.6773	0.248	0.5432	-	0.3368	0.0204	-	0.443	0.0008	-	0.0539	-	0.269	-	0.2119	-	0.4309	0.0039	0.4305	0.0974	0.075	0.1071	
		100 mM	0.2811	0.0745	-	-	0.0643	0.645	0.3037	0.3538	-	0.3858	0.0166	-	0.387	0.0068	-	0.0196	-	0.0048	-	0.0946	-	0.0519	6E-05	0.0542	0.0116	0.0219	0.0518	
	dG	10 mM	0.0545	0.0266	-	-	1E-05	0.6388	0.0332	0.9909	-	0.0003	0.3602	0.0038	0.0645	0.0579	-	0.002	0.0144	0.5274	-	0.0015	0.2518	0.0458	0.0005	0.0235	9E-05	0.0008	0.3101	
		20 mM	0.4602	0.0265	-	-	0.0009	0.9903	0.0147	0.4136	-	3E-05	0.9575	0.0036	0.0249	0.067	-	0.0006	0.0649	0.0725	-	0.0013	0.4081	0.0423	6E-05	0.6877	8E-05	0.0002	0.0668	
		50 mM	3E-11	0.0163	-	-	0.018	1E-11	5E-16	1E-06	-	6E-13	2E-05	2E-07	7E-16	0.001	-	3E-10	1E-13	0.0552	-	7E-14	1E-05	0.0019	0.0005	4E-11	5E-13	3E-12	2E-09	
		100 mM	4E-18	4E-05	-	-	0.0129	2E-15	2E-20	3E-08	-	4E-16	1E-07	4E-08	6E-20	1E-13	-	1E-10	6E-17	0.0122	-	2E-17	3E-06	0.0002	5E-05	1E-15	4E-15	3E-16	8E-20	

Table 4.13. Total p values for the SERS spectrum obtained from Beas-2b cells treated with selected inhibitors. Red cells show p value < 0.05, yellow highlight shows p value < 0.01, and green highlight shows p value < 0.001

		Raman Shift (cm ⁻¹)																											
		501	548	573	596	623	636	653	678	687	708	755	800	838	882	898	1002	1012	1030	1063	1130	1155	1180	1199	1218	1272	1317	1352	
Beas-2b	Aml	10 μM	7E-13	0.0049	-	-	6E-08	3E-09	2E-13	0.1886	-	2E-18	0.0001	7E-12	2E-17	0.2533	-	1E-15	5E-16	0.0008	-	4E-19	0.0654	3E-13	0.0101	7E-14	2E-16	3E-17	7E-11
		20 μM	3E-13	0.1497	-	-	3E-10	0.0047	2E-16	0.9137	-	9E-20	0.0063	0.0037	2E-14	0.0523	-	2E-14	4E-12	0.0184	-	1E-19	0.2779	6E-08	0.0006	2E-09	3E-15	1E-17	1E-09
		50 μM	4E-09	0.0053	-	-	5E-06	0.0001	4E-09	0.7029	-	6E-16	0.0005	1E-12	7E-11	0.0002	-	2E-14	5E-09	0.0193	-	3E-14	0.0381	1E-09	0.0668	4E-12	2E-14	1E-12	2E-06
		100 μM	1E-09	0.5355	-	-	2E-11	0.0003	2E-10	0.3229	-	2E-20	0.0008	3E-11	1E-11	0.3397	-	2E-14	5E-13	0.0007	-	5E-15	0.2165	6E-15	0.0031	3E-13	2E-15	5E-15	8E-10
	Cytod	1 μM	6E-11	0.3931	-	-	4E-11	0.0313	3E-07	0.0003	-	9E-14	0.6122	3E-11	8E-09	-	-	1E-16	6E-09	0.3405	-	2E-11	0.7816	3E-08	7E-05	0.0135	7E-14	2E-10	0.0024
		2 μM	4E-08	0.6704	-	-	2E-06	0.2869	1E-05	0.0085	-	2E-09	0.7246	6E-07	5E-06	-	-	6E-16	0.0003	1E-05	-	2E-06	0.752	6E-09	2E-07	0.822	2E-08	7E-07	0.0614
		5 μM	1E-19	3E-06	-	-	1E-06	4E-10	2E-18	0.0771	-	1E-24	2E-05	2E-14	3E-19	-	-	7E-14	3E-19	0.0011	-	1E-23	0.066	3E-12	0.0006	1E-08	2E-17	1E-17	1E-10
		10 μM	2E-10	0.8166	-	-	2E-09	2E-10	5E-13	0.0016	-	2E-22	2E-05	2E-16	1E-15	-	-	8E-14	1E-13	0.0001	-	5E-21	0.8452	5E-13	0.0006	2E-10	9E-16	2E-16	3E-08
	Sucrose	200 mM	0.1241	0.02	-	-	0.7014	0.0348	0.5671	-	-	0.4672	-	-	0.1213	-	-	0.846	0.6265	0.0294	-	0.2421	-	0.1832	-	0.5932	0.086	0.5434	0.0197
		300 mM	0.0004	0.1145	-	-	0.1552	0.0429	0.3356	-	-	0.8803	-	-	0.716	-	-	0.5395	0.1506	0.0115	-	0.7958	-	0.0572	-	0.075	0.4452	0.8438	0.2827
		400 mM	6E-05	0.3574	-	-	0.6038	0.4802	0.943	-	-	0.5863	-	-	0.9436	-	-	0.0782	0.0567	0.0056	-	0.6023	-	0.0261	-	0.017	0.9517	0.228	0.0139
		500 mM	8E-08	0.4232	-	-	0.0004	0.1937	0.0015	-	-	2E-09	-	-	0.1344	-	-	3E-11	0.3723	0.0741	-	0.0075	-	3E-07	-	0.0457	0.0104	0.0334	0.9342
	Nys	100 nM	0.0036	-	-	0.9527	-	0.0012	0.2858	-	-	-	0.1532	-	-	-	-	0.004	-	0.117	-	0.0009	-	-	-	-	0.0559	0.0708	0.0041
		200 nM	4E-05	-	-	0.823	-	0.1556	0.0966	-	-	-	0.8182	-	-	-	-	0.7838	-	0.0228	-	0.0266	-	-	-	-	0.658	0.6044	0.0008
		300 nM	9E-06	-	-	0.0912	-	0.3759	0.1429	-	-	-	0.0312	-	-	-	-	0.124	-	0.0632	-	0.0032	-	-	-	-	0.192	0.0347	0.0005
		400 nM	2E-09	-	-	0.1476	-	0.4447	0.0003	-	-	-	0.0006	-	-	-	-	0.1745	-	0.0233	-	9E-05	-	-	-	-	0.0036	6E-05	0.0003
	Fil	0.1 μM	1E-08	0.6209	-	0.032	-	0.7194	0.0001	0.1725	-	9E-12	-	3E-06	1E-05	0.3206	-	3E-11	0.0006	0.0013	-	4E-06	0.1365	4E-08	0.1628	0.9871	3E-07	1E-06	0.5489
		0.5 μM	9E-07	0.0436	-	0.0059	-	0.2779	0.5125	0.0018	-	2E-09	-	4E-07	0.0041	0.0971	-	2E-12	0.1529	0.0004	-	2E-05	0.1531	1E-09	0.0059	0.1008	7E-05	6E-06	0.3191
		1 μM	2E-09	0.5282	-	0.0531	-	0.0907	3E-06	0.0503	-	9E-10	-	7E-06	5E-07	0.4817	-	6E-14	2E-05	0.361	-	2E-09	0.9907	7E-07	0.9417	0.1683	8E-09	3E-10	0.0013
		2 μM	2E-08	0.7045	-	0.1564	-	0.0588	0.1095	0.0089	-	1E-12	-	0.0094	0.002	7E-05	-	1E-13	0.0099	0.0004	-	2E-06	0.6922	3E-09	0.009	0.3997	6E-07	6E-09	0.7597
	Dyn	10 nM	0.0594	-	-	0.8957	-	-	-	-	-	0.0644	0.3941	-	-	-	-	-	-	-	-	0.4784	-	0.322	-	-	0.7665	0.9284	0.291
		20 nM	0.0279	-	-	0.5076	-	-	-	-	-	0.2596	0.1193	-	-	-	-	-	-	-	-	0.6839	-	0.025	-	-	0.2308	0.7211	0.1403
		40 nM	0.0158	-	-	0.9774	-	-	-	-	-	0.0429	0.6958	-	-	-	-	-	-	-	-	0.6477	-	0.0119	-	-	0.7448	0.5963	0.0302
		80 nM	0.0003	-	-	0.0892	-	-	-	-	-	0.0007	0.6197	-	-	-	-	-	-	-	-	0.3156	-	0.0028	-	-	0.3303	0.2156	0.407
NaN3	10 mM	0.0001	0.0282	0.0079	0.7611	0.0073	0.0098	0.6206	0.0445	-	0.0001	-	0.0658	0.6976	0.2502	0.027	0.0004	0.2995	0.0361	-	0.0133	-	0.0003	0.0506	-	8E-05	0.1848	0.0687	
	20 mM	0.0008	0.2009	0.0469	0.3849	0.0005	0.0129	0.9301	0.0536	-	1E-05	-	0.0058	0.6903	0.1196	0.0062	9E-06	0.6193	0.0272	-	0.0137	-	6E-05	0.0333	-	4E-05	0.2449	0.0472	
	50 mM	2E-05	0.0178	0.0006	0.3516	0.0005	0.53	0.8056	0.1482	-	4E-06	-	0.0005	0.2371	0.1391	0.0004	1E-06	0.1062	0.1218	-	3E-05	-	0.0005	0.0036	-	2E-06	0.0001	0.7808	
	100 mM	0.001	0.2856	0.019	0.9613	0.0023	0.9193	0.73	0.1078	-	0.0003	-	0.0021	0.1698	0.3858	0.001	1E-06	0.1807	0.1714	-	0.0007	-	0.002	0.0031	-	8E-05	0.0011	0.1744	
dG	10 mM	3E-11	1E-05	-	0.0213	7E-06	1E-09	4E-17	0.0473	-	5E-23	1E-06	4E-16	1E-21	8E-09	-	7E-14	9E-19	1E-05	-	8E-22	0.0219	3E-12	6E-05	1E-14	1E-16	9E-16	4E-12	
	20 mM	1E-19	0.0002	-	0.0208	6E-08	1E-12	3E-19	0.0009	-	8E-31	3E-06	9E-17	2E-22	0.0006	-	8E-14	9E-20	0.0001	-	4E-25	0.0163	4E-14	4E-06	3E-13	5E-16	3E-18	4E-12	
	50 mM	3E-29	1E-06	-	0.0214	4E-05	9E-13	1E-16	0.054	-	3E-21	4E-07	5E-16	1E-21	0.0119	-	7E-14	3E-18	5E-06	-	9E-21	0.0032	8E-10	4E-05	3E-15	1E-14	6E-16	3E-12	
	100 mM	1E-15	0.2403	-	0.0116	2E-07	7E-13	3E-18	0.0012	-	8E-27	9E-07	1E-15	3E-15	4E-08	-	9E-14	7E-17	7E-06	-	5E-19	0.0907	2E-14	2E-05	4E-10	1E-16	8E-16	4E-12	

Table 4.14. Total p values for the SERS spectrum obtained from HeLa cells treated with selected inhibitors. Red cells show p value < 0.05, yellow highlight shows p value < 0.01, and green highlight shows p value < 0.001

		Raman Shift (cm ⁻¹)																											
		501	548	573	596	623	636	653	678	687	708	755	800	838	882	898	1002	1012	1030	1063	1130	1155	1180	1199	1218	1272	1317	1352	
HeLa	Nys	100 nM	0.3659	0.4006	-	-	-	0.4572	0.8375	0.0699	-	0.4928	-	-	-	-	-	0.19	-	-	-	0.6211	-	0.6123	-	-	0.5656	0.6488	0.3252
		200 nM	0.68	0.9412	-	-	-	0.2845	0.9593	0.0263	-	0.4117	-	-	-	-	-	0.2892	-	-	-	0.3294	-	0.3784	-	-	0.1134	0.4558	0.6058
		300 nM	0.4325	0.2753	-	-	-	0.0692	0.6476	0.3179	-	0.0677	-	-	-	-	-	0.0191	-	-	-	0.5962	-	0.0888	-	-	0.7828	0.5042	0.0791
		400 nM	0.0987	0.9717	-	-	-	0.8178	0.288	0.1912	-	0.4361	-	-	-	-	-	0.1562	-	-	-	0.9953	-	0.7224	-	-	0.5002	0.6626	0.6272
	Dyn	10 nM	0.0308	0.3727	-	-	0.0005	0.6625	0.1028	0.2467	-	0.0013	-	-	-	0.0787	-	-	-	-	-	0.4225	-	0.0134	-	0.7385	0.9083	0.7255	0.8988
		20 nM	0.1261	0.415	-	-	0.0882	0.7457	0.1732	0.5539	-	0.0339	-	-	-	0.1397	-	-	-	-	0.2083	-	0.1113	-	0.1917	0.3358	0.311	0.2068	
		40 nM	0.6021	0.0257	-	-	0.2566	0.4188	0.4069	0.1017	-	0.5633	-	-	-	0.0366	-	-	-	-	0.8473	-	0.4263	-	0.0783	0.6601	0.7537	0.3989	
		80 nM	0.0491	0.2605	-	-	0.019	0.1636	0.4452	0.0823	-	0.0198	-	-	-	0.0377	-	-	-	-	0.0412	-	0.0973	-	0.3627	0.1724	0.1744	0.1701	
	NaN3	10 mM	0.0005	0.2584	-	0.0396	0.0179	0.3858	0.3063	0.0038	-	0.0002	0.7983	3E-05	0.0013	0.729	0.0083	4E-05	0.0017	0.0003	0.232	0.0006	-	0.0013	3E-06	0.8169	0.0045	0.0006	0.0096
		20 mM	1E-06	0.4124	-	0.3875	0.0033	0.0532	5E-05	0.2477	-	1E-06	0.7013	1E-05	1E-05	0.8189	0.002	1E-07	0.0002	0.0322	0.0179	9E-06	-	0.0132	1E-07	0.7284	2E-05	0.0089	0.0003
		50 mM	5E-05	0.4311	-	0.2478	0.0049	0.0573	0.0138	0.182	-	2E-05	0.3293	0.0024	0.008	0.8314	0.0235	3E-06	0.0021	0.0002	0.0965	0.0012	-	0.0116	0.0013	0.1081	5E-05	2E-06	0.0074
		100 mM	8E-05	0.957	-	0.097	0.235	0.3495	0.3089	0.0202	-	0.0018	0.0883	7E-05	0.0033	0.9146	0.0036	9E-08	0.0279	0.0102	0.0201	0.0009	-	0.0083	0.0002	0.6126	0.0035	0.0008	0.0059
	dG	10 mM	0.0007	0.1913	-	-	1E-06	0.1024	0.3337	0.0021	-	1E-06	0.095	0.0001	0.0002	-	-	3E-06	0.1248	0.0009	-	0.0004	0.0019	9E-05	7E-09	0.163	0.0141	0.0049	0.2702
		20 mM	1E-11	0.6019	-	-	6E-09	3E-09	6E-11	0.3526	-	1E-10	0.7464	2E-12	6E-15	-	-	2E-08	4E-09	0.4769	-	5E-11	0.3169	0.0002	3E-10	2E-05	1E-09	3E-09	1E-07
		50 mM	2E-12	7E-06	-	-	8E-05	2E-12	4E-12	0.0063	-	4E-08	5E-06	4E-11	2E-16	-	-	1E-08	2E-13	2E-05	-	3E-12	0.0049	0.0021	6E-08	5E-12	2E-11	6E-11	7E-11
		100 mM	2E-12	3E-06	-	-	0.0004	3E-12	5E-12	0.0077	-	9E-09	2E-06	2E-11	3E-16	-	-	1E-08	3E-13	5E-05	-	3E-12	0.0003	0.0133	1E-07	3E-12	2E-11	5E-11	7E-11

Table 4.15. Total p values for the SERS spectrum obtained from PNT1A cells treated with selected inhibitors. Red cells show p value < 0.05, yellow highlight shows p value < 0.01, and green highlight shows p value < 0.001

			Raman Shift (cm ⁻¹)																											
			501	548	573	596	623	636	653	678	687	708	755	800	838	882	898	1002	1012	1030	1063	1130	1155	1180	1199	1218	1272	1317	1352	
PNT1A	Aml	10 μM	0.5382	0.4788	-	-	0.1998	0.0381	0.0955	0.0431	-	0.3963	0.0713	-	0.6848	0.8089	-	0.7466	0.5268	0.8908	-	0.8623	-	0.5952	-	0.0961	0.926	0.6082	0.049	
		20 μM	0.0644	0.079	-	-	0.8845	0.0875	0.3656	0.0212	-	0.0013	0.3902	-	0.269	0.085	-	2E-05	0.2288	7E-05	-	0.0071	-	0.0099	-	0.5304	0.0309	0.0048	0.0047	
		50 μM	0.0139	0.2106	-	-	0.0408	0.1827	3E-05	0.1443	-	0.5642	0.7592	-	0.0039	0.0065	-	0.0487	0.0042	0.0435	-	0.0016	-	0.3871	-	0.1999	0.0099	0.0407	0.0408	
		100 μM	0.1406	0.8137	-	-	0.1729	0.1566	0.2377	0.3159	-	0.0024	0.3459	-	0.3699	0.8162	-	0.0079	0.2028	0.0792	-	0.1817	-	0.9364	-	0.9985	0.0833	0.0909	0.0486	
	Sucrose	200 mM	1E-07	2E-05	-	-	0.321	3E-07	2E-09	0.0026	-	0.0001	0.0025	0.0001	2E-10	0.0042	-	0.0006	1E-07	2E-05	-	8E-10	0.0005	0.7473	0.0153	2E-06	9E-08	2E-09	3E-09	
		300 mM	9E-07	0.0606	-	-	0.0012	3E-05	4E-06	0.0836	-	1E-09	0.0095	7E-07	2E-06	0.5383	-	1E-05	9E-06	0.0037	-	1E-06	0.0693	0.4499	0.038	5E-06	4E-08	3E-07	2E-06	
		400 mM	3E-14	2E-07	-	-	0.0103	7E-14	3E-15	0.0001	-	9E-10	6E-06	6E-09	2E-14	0.5103	-	7E-07	3E-13	1E-05	-	2E-14	0.0002	0.1845	0.0051	4E-10	4E-10	1E-12	2E-12	
		500 mM	1E-16	5E-08	-	-	0.0008	4E-16	4E-16	9E-05	-	3E-13	2E-07	4E-11	1E-17	0.1804	-	9E-08	2E-15	8E-09	-	2E-17	5E-05	0.1027	0.0073	7E-15	5E-14	6E-16	1E-15	
		Cpm	10 mM	0.02	0.0365	-	-	4E-05	0.0889	3E-08	0.0435	-	3E-06	0.3478	0.0001	0.0022	0.2104	-	3E-06	0.0003	0.1412	-	8E-06	0.736	0.2099	0.0337	0.009	0.0187	0.0002	0.005
	20 mM		0.2149	0.6799	-	-	0.1475	0.1797	0.8399	0.9428	-	0.1112	0.0378	0.3918	0.1337	0.5608	-	0.0036	0.1178	0.2224	-	0.355	0.1749	0.0082	0.0536	0.565	0.5399	0.4042	0.072	
	50 mM		1E-13	7E-06	-	-	0.0023	6E-06	2E-14	0.0098	-	1E-06	0.0049	6E-08	2E-08	0.3875	-	6E-06	3E-15	0.0116	-	3E-12	0.0002	0.7778	0.6113	1E-07	2E-13	5E-12	1E-07	
	100 mM		2E-16	6E-07	-	-	0.088	4E-09	4E-16	1E-05	-	0.0011	0.4496	1E-08	4E-18	0.2186	-	7E-08	1E-14	2E-09	-	5E-17	0.0007	0.2079	0.1155	4E-08	8E-14	9E-16	8E-16	
	Nys	100 nM	0.0138	-	-	-	-	0.0266	0.6351	0.0641	-	0.0034	-	-	0.0843	-	-	0.0044	0.1994	0.5309	-	0.0346	-	0.0717	-	0.1046	0.0014	0.1152	0.3526	
		200 nM	0.2653	-	-	-	-	0.1773	0.0611	0.9341	-	0.9728	-	-	0.122	-	-	0.5693	0.1095	0.0998	-	0.126	-	0.8845	-	0.6813	0.2227	0.1958	0.0797	
		300 nM	0.3307	-	-	-	-	0.1566	0.1618	0.084	-	0.7739	-	-	0.0848	-	-	0.3299	0.0879	0.0739	-	0.1393	-	0.385	-	0.0712	0.2543	0.1898	0.3442	
		400 nM	0.0455	-	-	-	-	0.0897	0.054	0.9627	-	0.1474	-	-	0.1099	-	-	0.0027	0.2685	0.6517	-	0.0119	-	0.1197	-	0.5933	0.1854	0.0271	0.1132	
	Fil	0.1 μM	0.0003	0.0706	-	-	0.0003	0.0597	0.0003	0.6202	-	0.0003	0.7997	-	8E-05	0.0097	-	0.0001	0.0144	0.6665	-	0.0032	0.37	0.2956	0.0018	0.3425	0.0148	0.0003	0.0236	
		0.5 μM	0.0009	0.0251	-	-	0.0968	5E-05	8E-05	0.0759	-	0.0002	0.3236	-	6E-05	0.0032	-	0.0024	0.0017	0.0179	-	4E-05	0.3433	0.6552	0.0421	0.0215	0.0011	3E-05	5E-05	
		1 μM	0.0078	0.0105	-	-	0.0432	0.055	7E-05	0.03	-	0.0002	0.2509	-	0.0056	0.0515	-	0.0002	0.0144	0.5031	-	0.0012	0.6813	0.1282	0.0111	0.0336	0.0012	0.002	0.0557	
		2 μM	4E-07	0.0002	-	-	0.2842	8E-09	2E-11	0.0015	-	1E-06	0.0002	-	6E-11	5E-08	-	2E-06	2E-09	0.0002	-	3E-10	0.0128	0.6016	0.0446	6E-06	2E-06	1E-08	4E-10	
	NaN3	10 mM	0.0343	0.2797	0.0422	0.1344	-	0.0452	0.5543	0.9636	-	0.6169	0.04	-	0.5667	0.2922	-	0.1573	0.7648	0.0033	-	0.2598	-	0.0002	-	2E-05	0.3602	0.1201	0.0714	
		20 mM	0.2845	0.3172	0.3267	0.3322	-	0.3554	0.3374	0.3159	-	0.3262	0.3383	-	0.3253	0.3286	-	0.3955	0.3357	0.3719	-	0.3006	-	0.3585	-	0.3871	0.3172	0.5489	0.2398	
		50 mM	0.2254	0.3122	0.3309	0.3165	-	0.3317	0.3089	0.3105	-	0.3266	0.338	-	0.2812	0.3309	-	0.4049	0.2995	0.3188	-	0.2463	-	0.3639	-	0.3818	0.2883	0.0045	0.9942	
		100 mM	0.2354	0.3132	0.3314	0.32	-	0.3324	0.3138	0.3114	-	0.326	0.339	-	0.2849	0.2175	-	0.417	0.3031	0.3244	-	0.2498	-	0.3655	-	0.3855	0.2904	0.0066	0.5405	
	dG	10 mM	0.0019	0.0932	0.1434	0.0678	0.0002	0.7719	0.0047	0.2599	0.0381	0.0059	0.217	0.0004	0.0169	-	-	0.002	0.0052	0.3266	-	0.0002	0.1492	0.0116	0.0002	0.2303	0.0018	0.0001	0.0769	
		20 mM	8E-05	0.8813	0.6011	0.784	2E-06	0.0635	3E-06	0.449	0.0347	2E-07	0.0067	0.0004	0.0002	-	-	2E-05	0.0034	0.3686	-	9E-06	0.2361	0.0778	8E-06	0.4281	0.0002	2E-05	0.0555	
		50 mM	0.0013	0.0005	0.1175	0.084	0.011	0.0459	2E-06	0.0011	0.0027	3E-07	0.1092	5E-05	3E-06	-	-	4E-06	1E-05	0.0045	-	1E-07	0.0319	0.1487	0.0015	0.0057	7E-05	2E-05	8E-05	
		100 mM	3E-10	0.0004	0.0316	0.0521	7E-05	8E-07	1E-10	3E-05	0.2312	2E-14	3E-05	4E-07	6E-12	-	-	2E-07	2E-10	3E-07	-	5E-13	0.152	0.0121	0.0039	2E-11	6E-10	7E-13	3E-09	

4.4. ENDOCYTOSIS DEVELOPMENT STUDIES

4.4.1. Endosome Isolation and Characterization

In this study, endosomes were isolated in the presence and absence of AuNPs and isolated endosomes were characterized by DLS and SEM as shown in Figure 4.65., Table 4.16. and Figure 4.66. In Figure 4.65., hydrodynamic sizes of endosomes in the presence (AuE) or absence of AuNPs (CtrlE) were given. In both of the endosomes, there are three different populations where first peak has average 38 nm size for CtrlE and 32 nm size for AuE, and the second peak has average 454 nm size for CtrlE and 720 nm size for AuE, and the third peak has average 4.6 μm size for CtrlE and 4.7 size μm for AuE. 3 different size populations could be obtained due to the different sizes of different type of endosomes. When endosomes were newly formed, they have 60-120 nm size as coated vesicles and early endosomes have 100-500 nm sizes. During the maturation process intraluminal vesicles (ILVs) with 40-100 nm sizes were formed and late endosomes possess 250-1000 nm size with multiple ILVs in the structure. After lysosomal fusion, endolysosomes thought to have sizes bigger than 1 μm [485,486]. On the other hand, macropinosomes could have a size range between 0.2-10 μm in diameter which is generally larger than endosomes [183,184]. Thus, first peak could be related with newly formed coated vesicles or ILVs and even early endosomes could be found in the first peak because it has a size range between 10-100 nm. Second peak could be related with the late endosomes and last one could be coming from the lysosomes, endolysosomes and macropinosomes. It should be also noted that, to observe the early endosomes, coated vesicles or any other early step components of endocytosis, AuNP were remained in the cell culture media for 24 h. This will lead AuNP endocytosis in different time points which will provide formation of different endosomal compartments in different time points.

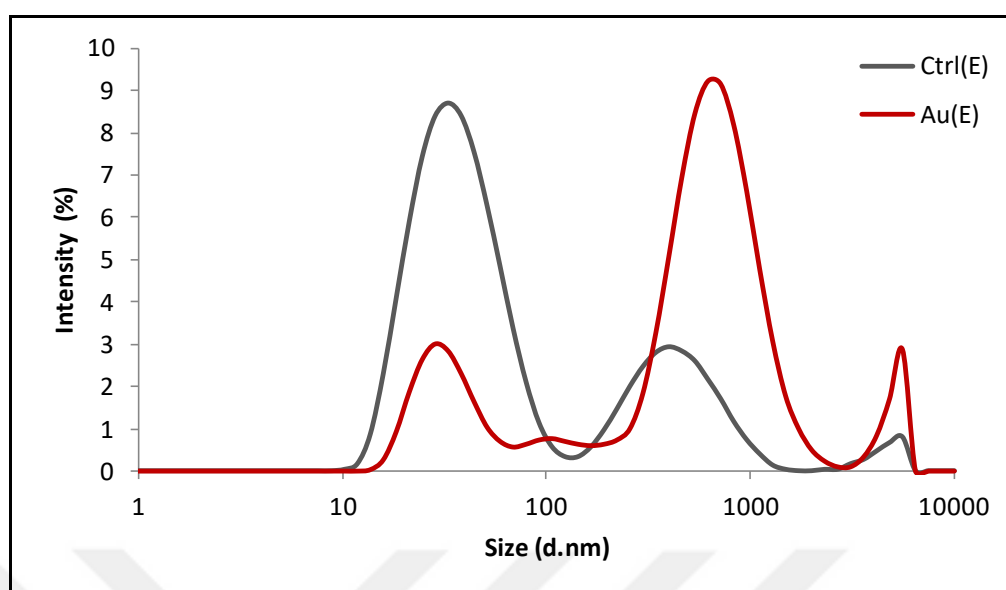


Figure 4.65. DLS spectrum of isolated endosomes in the absence (CtrlE) and presence (AuE) of AuNPs with 24 h treatment

Furthermore, peak distributions were found as changed when endosomes were filled with AuNPs as seen in Table 4.16. and intensity of the first peak shifted from 72 percent to 17 percent and second peak shifted from 25 percent to 72 percent. It could be caused by the AuNPs endocytosis because as seen in the Figure 4.67., endosomes without AuNPs do not show late endosome markers and when endosomes were filled with AuNPs, late endosomes were started to observe and with 24 h treatment of AuNPs like in the AuE samples, late endosomes were observed most. Besides, lysosomes were found more when compared to the control endosomes. Thus, shift of the peak distributions could be caused by the AuNPs presence inside of the endosomes.

Table 4.16. Size properties of isolated endosomes

Peak No	CtrlE		AuE	
	Size (d.nm)	Intensity (%)	Size (d.nm)	Intensity (%)
1	38.59 ± 1.37	72.2 ± 1.01	32.6 ± 4.18	17.36 ± 1.35
2	454.26 ± 62.8	25.43 ± 0.92	720 ± 79.2	72.23 ± 2.97
3	4623 ± 277.5	2.33 ± 0.15	4718 ± 63.6	6.96 ± 0.68

Sizes of the endosomes were also characterized by SEM as seen in Figure 4. 66. As seen, for control endosomes without AuNPs treatment (CtrlE), mostly small spherical structures were observed which is consistent with obtained hydrodynamic size measurements. In DLS measurements, most of the particles were found smaller than 100 nm which could be attributed to early endosomes, ILVs or newly formed coated vesicle. On the other hand, for AuNPs filled endosomes (AuE), bigger spherical structures were observed in SEM images and it could be related with the distribution of the particles observed in DLS measurements in which 70 percent of particles have size range between 200-2000 nm and 7 percent of the population have size bigger than 3 μm which could be attributed to late endosomes, lysosomes, endolysosomes and macropinosomes. Thus, smaller particles could not observe for AuE endosomes while mostly large structures were observed.

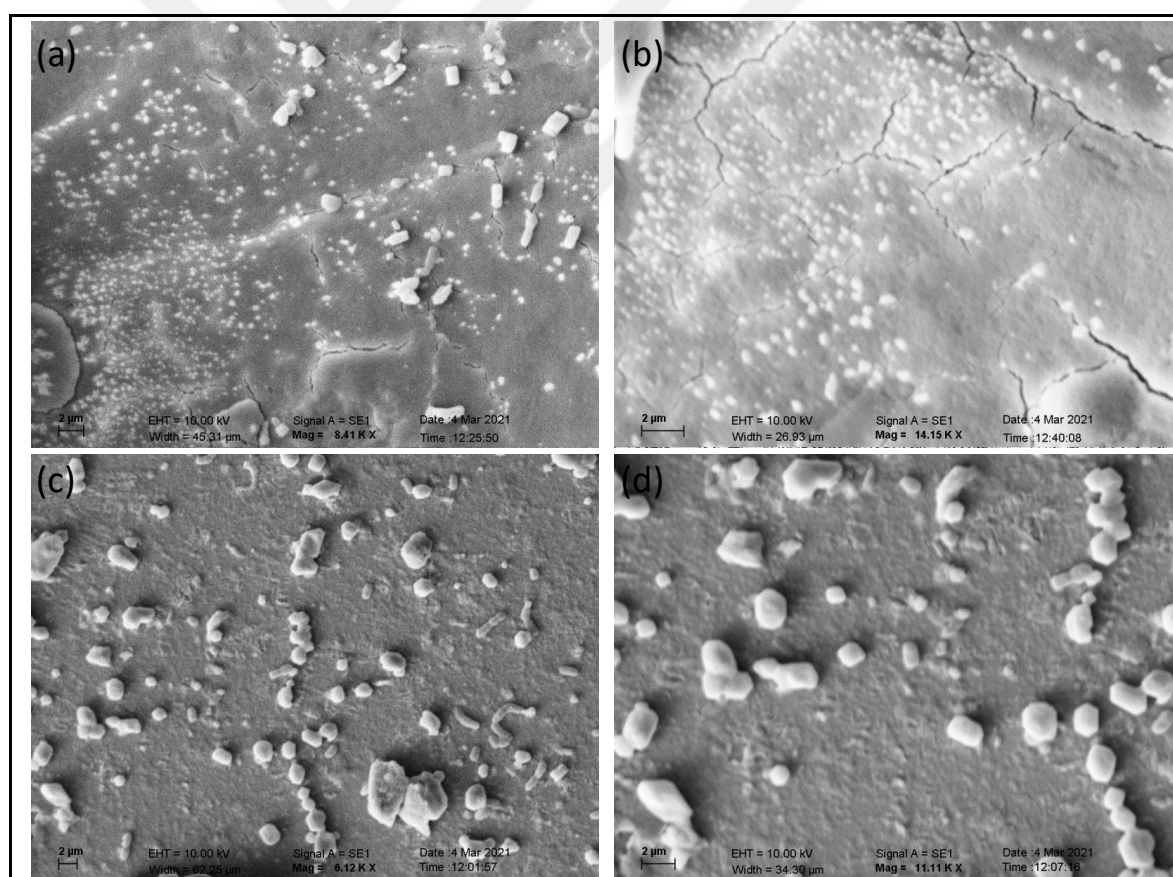


Figure 4.66. SEM images of isolated endosomes (a), (b) without AuNPs and (c), (d) with AuNPs

4.4.2. Western Blotting

Characterization of isolated endosomes was also achieved by immunoblotting. Figure 4.67. shows expression levels of different organelle markers: Early Endosomal Antigen 1 (EEA1, EE marker), Ras-associated binding protein 7 (Rab7, LE marker), Lysosomal-associated membrane protein 1 (LAMP1, Lysosomal marker), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, cytoplasmic marker), Calnexin (ER marker) and Golgi matrix protein 130 (GM130, Golgi marker). As seen, in the control endosomes (CTRL) early and late endosome marker expressions were not observed and lysosomal marker expression was seen with GAPDH, calnexin and GM130 expressions. In CTRL samples, early and late endosomes would not be observed which is probably caused by having a smaller number of endosomes and no stimuli to enhance endocytosis like AuNPs. For the investigation of time dependent endosomal expression levels, endosomes were collected at different AuNPs treatment points such as 15 min, 30 min, 1 h, 4 h, 16 h and 24 h. As seen, with increasing time, EEA1 expression decreased and Rab7 expression increased. This expression levels showed that AuNPs endocytosis continues up to 4 h and after then, internalization of new AuNPs stops and they are started to accumulate in the late endosomes starting from 4 h. Thus, after 4 h, early endosome formation was not observed despite already uptaken AuNPs were started to accumulate in late endosomes. On the other hand, lysosomal marker expression was achieved in all time points with increasing expression for 4, 16 and 24 h when compared with AuNPs treated endosomes. Increase in the LAMP1 expression with increasing time for 4, 16 and 24 h is probably caused by the translocation of AuNPs found in early endosomes to the endolysosomes. Thus, when early endosome expression is stopped, late endosome and endolysosome marker expressions were observed. On the other hand, for the expression of LAMP1 in other time points, it could be observed that LAMP1 expression is highest for 15 min compared with 15 min-1 h treatments and expression levels decreases with increasing time up to 1 h. This could be explained by two different mechanisms. Lysosomal marker expression was found similar with the CTRL endosomes; thus, it could be concluded that lysosomal marker expression in short times could be unrelated with AuNPs internalization and caused by the unstimulated endocytosis pathway in the absence of AuNPs. On the other hand, it could be caused by the missing the late endosome formation and thus expression. Although AuNPs uptaken was observed by EEA1 expression, Rab7 expression could be missed at short

times due to the lower expression levels when compared with longer time treatments of AuNPs and uptaken AuNPs could be already find in endolysosomes as observed in LAMP1 expression.

Furthermore, contaminations of the obtained fractions with other cellular components were checked with organelle markers: GAPDH as cytoplasmic marker, calnexin as ER marker and GM130 as Golgi marker. It was found that GAPDH as cytoplasmic contaminant was found in all isolated endosomal fractions. It was an expected situation to observe GAPDH expression in the samples because it is known that GAPDH is found in the cytosol, nucleus, mitochondria and small vesicular fractions with various functions. On the other hand, it should be considered that GAPDH is associated with microtubules, vesicle trafficking, membrane fusion and cytoskeleton [487]. Most of these mechanisms are involved in the endocytosis pathway which means GAPDH should be found in the endocytic site surrounding the endosomal vesicles and it could be expected to observe GAPDH expression when endosomes were isolated by density gradient. Moreover, ER and Golgi markers were also observed in some of the isolated endosomes. Contaminations could be the result of the selected isolation method. To get pure isolated endosomes without any organelle contamination, several protocols and studies have been reported in the literature including fluorescence-activated organelle sorting [488,489], flow field-flow fractionation [490], immunoisolation with antibodies [491], magnetic bead based isolation [492,493], however, most of these methods require interference with the endosomal structure and in the case of investigation of the endosomal SERS signal obtained from uptaken AuNPs, any interference could cause misunderstanding the obtained SERS signal while trying to understand the source of SERS signal. Thus, traditional method based on density gradient-based ultracentrifugation was chosen and this selection could lead some contaminations coming from organelles associated with endocytosis. Besides, it could be also taken into account that, to get a SERS signal, there should be AuNPs association with organelles or vesicles and obtained isolated fractions were washed three times which means there was not any AuNPs in the surrounding environment which could be associated with ER or Golgi. Consequently, obtained SERS signal could be coming from AuNPs-filled endosomes not from Golgi and ER if there is any SERS signal.

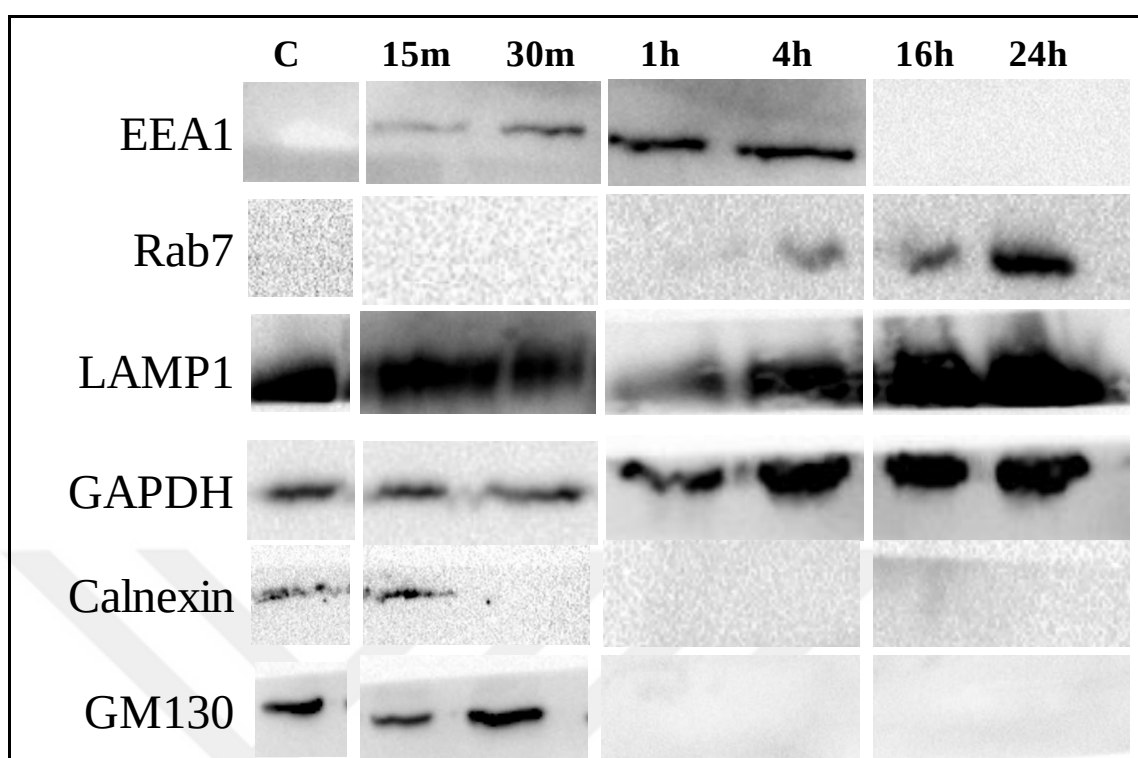


Figure 4.67. Western blotting results of isolated endosomes treated without AuNPs (C) or 15 min, 30 min, 1 h, 4 h, 16 h, 24 h AuNPs

After characterization of isolated endosomes, obtained expression levels were compared with cellular lysates as seen in Figure 4.68. Also, two different AuNPs treatment methods compared as 24 h treatment and 2 h pulse 22 h chase treatment of AuNPs. When comparing with cellular lysates, LAMP1 expression was found as enhanced in isolated endosomes while Rab7 expression was found as higher in 24 h treated whole cell samples. On the other hand when treatment types were compared, it was found that giving AuNPs for 2 h and then removing all AuNPs from the cellular medium provides accumulation of most of the AuNPs in the endolysosomes or lysosomes as reported in the literature [311,335].

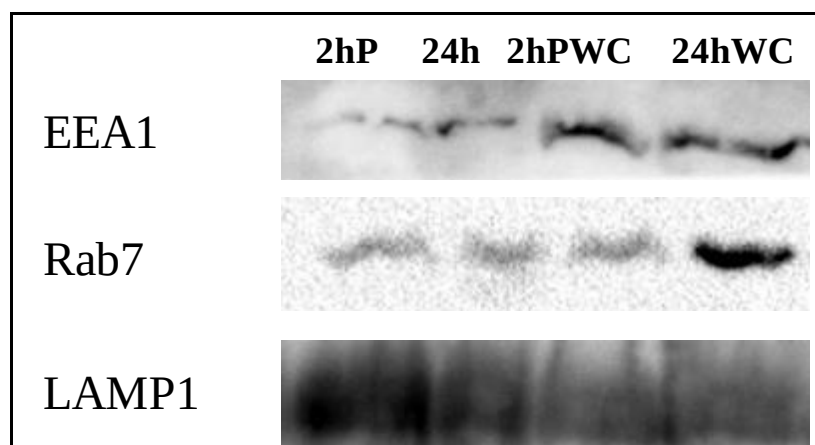


Figure 4.68. Western blot results of isolated endosomes with 2 h pulse (2hP) and 24 h (24h) treatment of AuNPs and whole cell 2 h pulse (2hPWC) and 24 h AuNPs treatment (24hWC)

4.4.3. Time Dependent AuNPs Uptake

Internalization of AuNPs by HeLa was investigated as time dependent manner for the explanation of SERS spectra obtained from isolated endosomes. SSC shift results after AuNPs internalization are shown in Figure 4.69. As seen; when AuNPs treatment time is lower than 4 h; enough granulation could not achieve to increase SSC shift in the cells. On the other hand, with 4, 16 and 24 h treatment of AuNPs, significant amount of AuNPs were uptaken by HeLa cells which is characterized by significant increase of SSC shift. When pulse-chase method was examined for the internalization of AuNPs, it was found that it has similar results with 1 h AuNPs treatment. Because, in the pulse-chase method, HeLa cells exposed to AuNPs for 2 h, then chased without AuNPs for 22 h. Thus, internalized amount was found as similar with 1 h treatment.

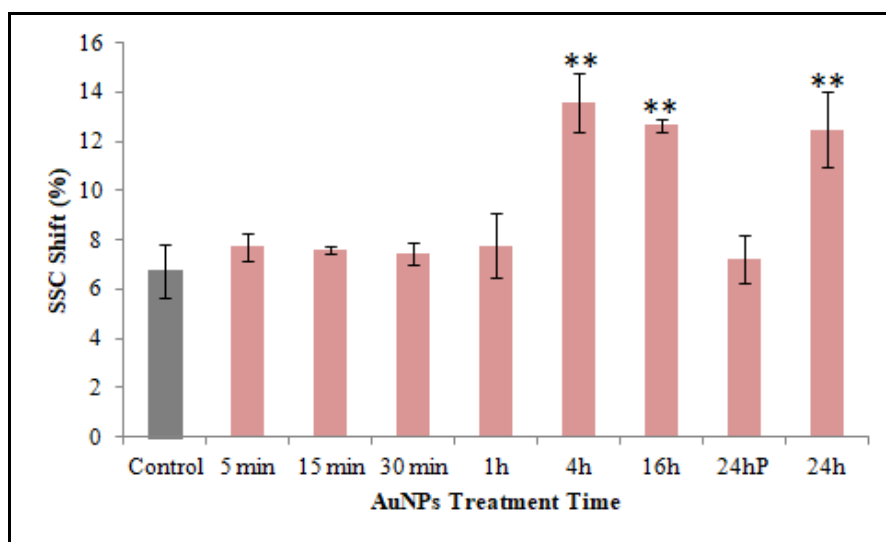


Figure 4.69. Time dependent AuNPs uptake by HeLa cells

4.4.4. SERS Measurements

For the investigation of endosomal spectral changes, SERS spectra of isolated endosomes were obtained for 15 min, 30 min, 1 h, 4 h, 16 h, 24 h, and 2 h pulse AuNPs treatment as seen in Figure 4.70. Time dependent changes were observed in the obtained SERS spectra of isolated endosomes and it was found that in lower treatment time points; spectra is similar with the control which is homogenization buffer used for isolation. SERS spectra of used solutions were shown in Figure 4.71. as control experiment and it can be seen that sucrose (Scr) interfere with obtained SERS spectra when there is not any AuNPs treatment. Furthermore, homogenization buffer gives same SERS signal with Scr because it includes 0.25 M sucrose, 3 mM Imidazole and 0.5 mM EDTA and only Scr gives SERS signal at used concentrations. Scr signal was found in the isolated endosomes, because even isolated endosomes were washed after isolation; when strong SERS signal could not achieve from the endosomes due to the lower amount of AuNPs internalized by cells; homogenization buffer signal was obtained from endosome spectra. On the other hand, with increasing time, distinct spectral bands were observed which are not found in not only homogenization buffer but also whole cell SERS spectra. It was realized that new SERS peaks were observed attributed to proteins at 523 cm^{-1} (S-S stretch of proteins), 662 cm^{-1} (Cystine), 906 cm^{-1} (Tyr), 951 cm^{-1} , (Proteins), 1235 cm^{-1} , 1281 cm^{-1} (Amide III), 1558 cm^{-1} (Trp) , 1600 cm^{-1} (C=O stretch, Amide I) and lipid structures at 972 cm^{-1} (Lipid),

1060 cm^{-1} (Chain C-C stretching of lipids), 1444 cm^{-1} (Cholesterol). The reason that obtained SERS spectra is composed of peaks corresponding to proteins and lipids is behind the nature of endosomes. Due to the being a part of cellular membrane, endosomes consist of lipid bilayer and protein structures on this lipid bilayer. Besides, endosomal pathway requires various proteins, as mentioned in previous chapter, for the development of the pathway and transfer of the cargo. Thus, when AuNPs uptaken by endosomes and AuNPs located inside the endosomes, mostly proteins and lipid structure of endosomal membrane come close vicinity to the AuNPs and this can be observed in the obtained SERS spectra as peaks corresponding to proteins and lipids. On the other hand, these new peaks show that; when endosomes were isolated from the cells, unique peaks attributed to the endosomes can be observed by SERS. Furthermore; when time dependent changes were compared, it was found that with increasing time, SERS spectra changes and starts to not resemble with the control spectra and show unique spectral bands mentioned above.

Time dependent changes can relate to the amount of AuNPs localized in endosomes. When uptaken amount of AuNPs internalized by cells were investigated, it was found that significant AuNPs uptake was obtained after 4 h treatment and this significant increase observed in the obtained SERS spectra with the arising new peaks over time. Spectral bands attributed to proteins and lipids also observed at small exposure times but with increasing time, intensity of the obtained bands increased with increased uptake of AuNPs. Although time dependent changes of the SERS spectra is directly related with the AuNPs uptake, it could be also related with the changed protein profile and behavior of endosomes with increasing time. When protein profile was examined by Western-Blotting (Figure 4.67.), it was realized that after 1 h, late endosome formation was observed and expression of lysosomal proteins increase. This situation can directly affect obtained SERS signal because, with the maturation of endosomal pathway, late endosomes are formed and then fuse with lysosomes. In the maturation process, multivesicular bodies are formed before late endosome maturation and cargo is localized in this MVBs [494]. Localization inside the MVBs can change aggregation status of AuNPs which is among the important parameters affect obtained SERS spectra [495]. With the change in the aggregation status of AuNPs and increase the AuNPs amount inside the endosomes, intensity of the obtained SERS signal can be increased and spectral bands corresponding to the endosomal structures including proteins and lipids can be observed.

It should be also noted that, some of the spectra spectral bands observed in the endosomes were similar with cellular SERS spectra such as 501 cm^{-1} (S-S), 623 cm^{-1} (C-C), 636 cm^{-1} (C-S), 882 cm^{-1} (Proteins), 1002 cm^{-1} (Phe), 1155 cm^{-1} (Proteins), 1180 cm^{-1} (Tyr, Phe), 1199 cm^{-1} (Trp), 1355 cm^{-1} (β -sheet). Obtained cellular peaks were attributed to the proteins. Thus, it can be said that in the cellular SERS spectra, mentioned protein attributed bands are directly coming from endosomal protein structures

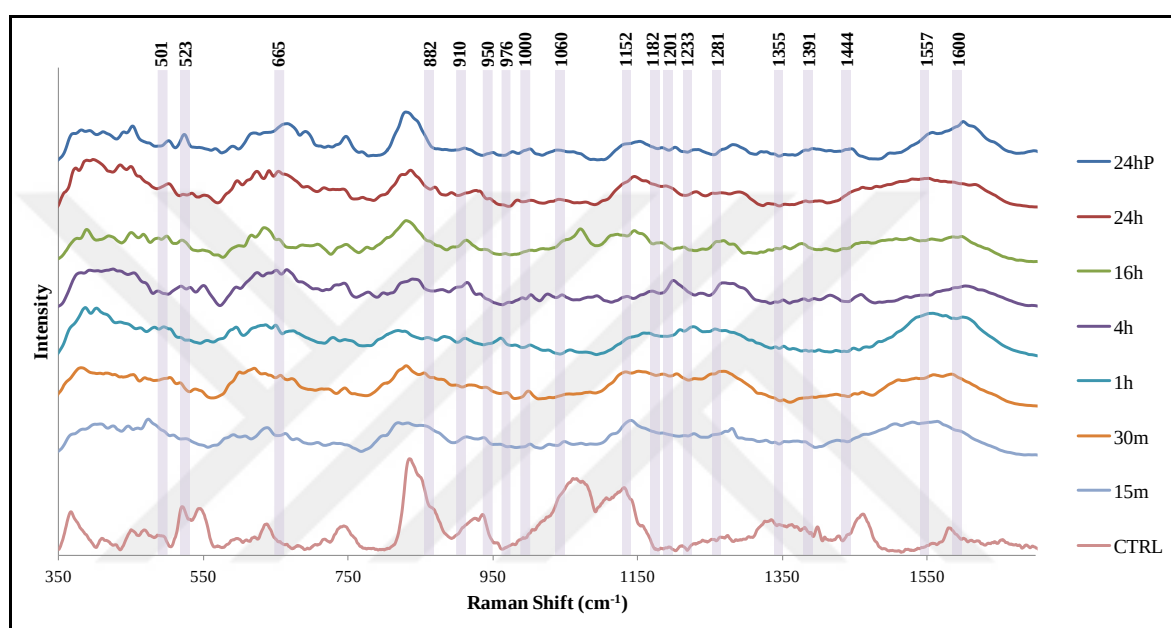


Figure 4.70. Average SERS spectra of isolated endosomes in different time points including 15 min, 30 min, 1 h, 4 h, 16 h, and 24 h, 2 h pulse (24hP) AuNP treated endosomes. CTRL: Homogenization Buffer

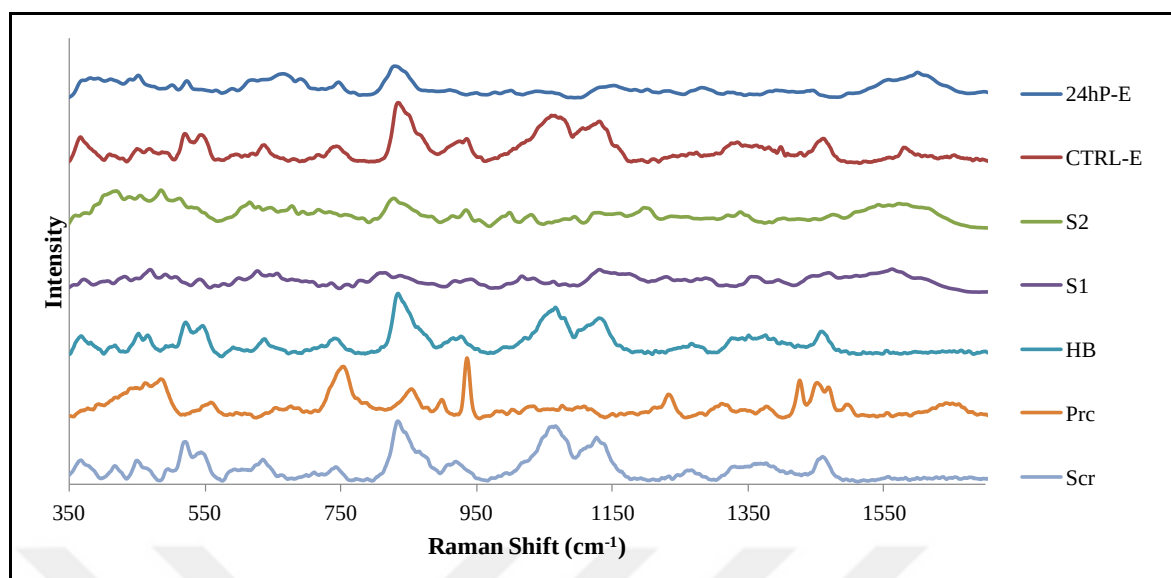


Figure 4.71. SERS spectra of sucrose (Scr), percoll (Prc), homogenization buffer (HB) with first supernatant including cytoplasmic fraction (S1), second supernatant including nucleus fraction (S2), endosome without AuNP treatment (CTRL-E) and endosome with 2 h pulse AuNPs treatment (24hP-E)

After the investigation of SRS spectra from isolated endosomes, it was compared with the cellular SERS spectra and also other cellular fractions as seen in Figure 4.72. While the isolations of endosomes; cytosolic fractions (C) were collected as first supernatant after the cell membrane is broken with a syringe. After the collection of cytosolic fraction, nuclear fraction (N) was separated from the endosomal fraction with sucrose-Percoll gradient in ultracentrifuge. SERS spectra of these fractions were measured and compared with endosomal and cellular SERS spectra in time dependent manner. As seen in the endosomal SERS spectra; when time is lower than 4 h, obtained SERS spectra from fractions and cells has lower signal, on the other hand after 4 h treatment AuNPs, important spectral changes were observed at each spectra. When cells were treated with AuNPs for 15 min, cellular spectra shown only spectral band corresponds to proteins (882 cm^{-1} , 1002 cm^{-1} , 1155 cm^{-1} , 1180 cm^{-1} , 1199 cm^{-1} and 1235 cm^{-1}). Moreover, SERS spectra of endosomal, nuclear and cytosolic fractions are similar with the control spectra due to the low amount or absence of AuNPs in the fractions. Even spectral changes are not clearly observed in the fractions, spectral bands at 524 cm^{-1} (S-S), 662 cm^{-1} (Cystine), 951 cm^{-1} (α -helix), 1281 cm^{-1} (Amide III) and 1444 cm^{-1} (Cholesterol) were observed in the fractions. Any spectral band observed in the cytosolic or nuclear fraction proves that 50 nm AuNPs is capable of

escaping endosomes and localize in different parts of the cells even at 15 min treatment. Figure 4.73. shows color of the isolated fractions with the comparison of control group and from those images it can be also seen that, color change caused by AuNPs when compared with the control can be observed in the samples which shows AuNPs presence in different cellular fractions.

In the 15 min treatment, peak at 524 cm^{-1} corresponding to S-S disulfide stretching in proteins was found in the cytosolic fraction and peak at 951 cm^{-1} corresponding to α -helix of proteins was found in nuclear fraction which shows after escaping the endosomes AuNPs interact with both cytosol and nucleus of the cell.

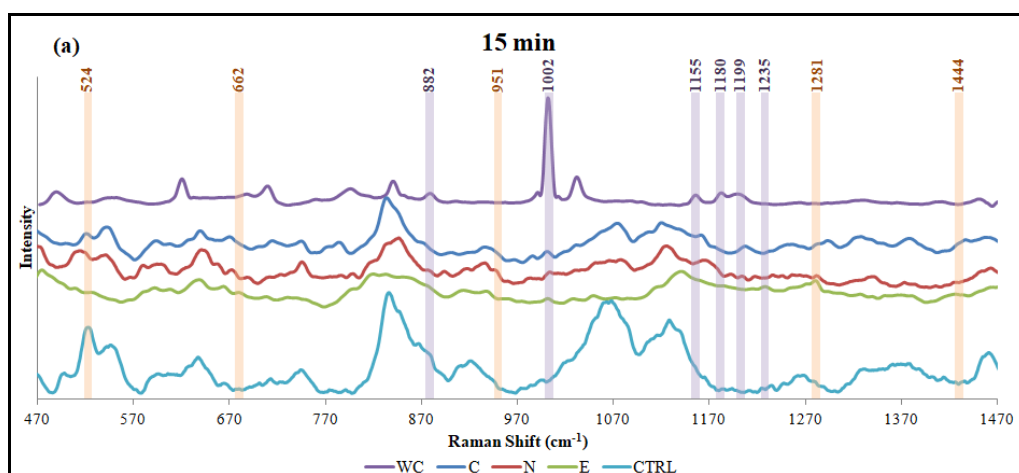
When AuNPs treatment increased up to 30 min, cellular spectra did not change while endosomal and cytosolic spectra seem different from the control group. In the endosomal and cytosolic spectra, spectral bands were found as same at the 15 min treatment which is attributed to protein structures including S-S disulfide bond, α -helix, Amide III and lipids with cholesterol. Obtained nuclear fractions did not give SERS signal and found similar with SERS spectra of the control group while still peaks at 662 cm^{-1} and 951 cm^{-1} can be observed in the SERS spectra.

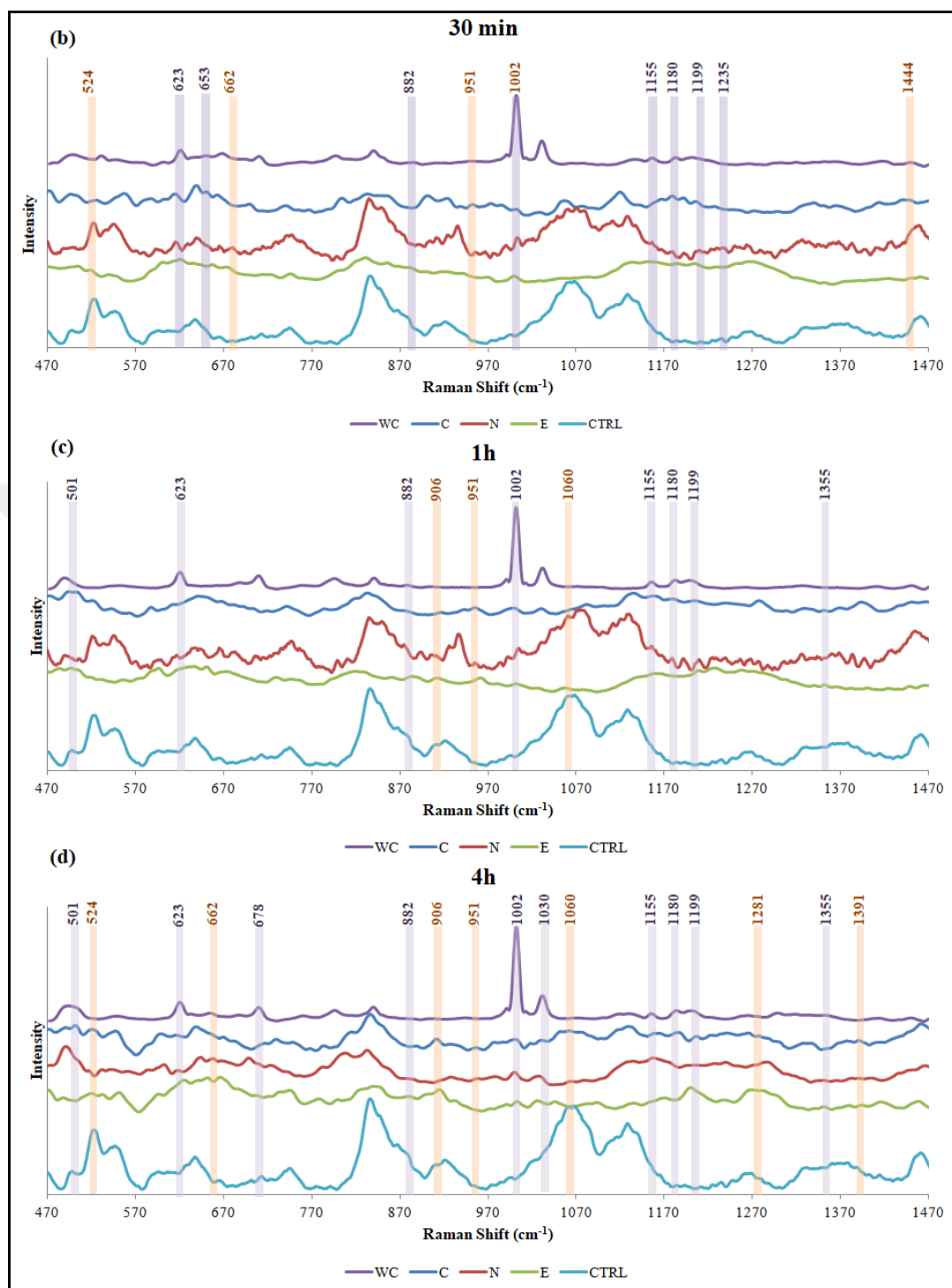
With 1 h AuNPs treatment, similar results were observed with 30 min treatment. SERS spectra of nuclear fraction were similar with control group while nuclear and endosomal fractions have different pattern in the SERS signal. With 1 h, different from the previous spectra, peaks at 501 cm^{-1} (S-S) and 1355 cm^{-1} (β -sheet) observed in the cellular SERS spectra and peaks at 906 cm^{-1} (Tyr), 1060 cm^{-1} (Lipids) were found in the endosomal fraction.

With 4 h AuNPs treatment; resemblance to the control group in each fraction is lost and unique spectral pattern in the fractions were more easily observed. Similar peaks were observed with 1 h treatment while a new peak at 1391 cm^{-1} (C-H) was also observed. When treatment time increased up to 16 and 24 h, similar peaks were observed with 1 and 4 h which corresponds to proteins and lipid structures. AuNPs localization can be also seen in the images of isolated fractions in Figure 4.72 as purple color after 1 h treatment in all fractions.

Time dependent spectral changes are directly related with the amount of AuNPs in the samples and also development of endosomal pathway. As mentioned before, AuNPs amount inside the cells increased significantly after 4 h treatment. Thus, before 4 h treatments, obtained cellular spectra and also fractional spectra do not give much information even there are still some spectral bands to observe. When the amount of AuNPs inside the cell increased, escaped AuNPs provided SERS signals in the cytosolic and nuclear fractions with detectable spectral bands. On the other hand, when endosomal protein profile is investigated, it can be also seen that late endosome formation was observed after 4 h treatment of AuNPs and endolysosome amount also increased. With that development, environment of the AuNPs changed and AuNPs become more aggregated which provided better SERS signal inside the endosomes.

This data show that, obtained cellular SERS spectra does not only consist of the AuNPs inside the endosomes when 50 nm AuNPs were used. AuNPs could escape from the endosomes and interact with the cytosolic and nuclear biomolecules and when these molecules come close vicinity to the AuNPs, their SERS signal can be reflected. Escape of the AuNPs could be even tracked at 15 min treatment with distinct SERS peaks at cytosolic and nuclear fractions such as 524 cm^{-1} for cytosolic fraction and 662 cm^{-1} , 951 cm^{-1} for nuclear fraction.





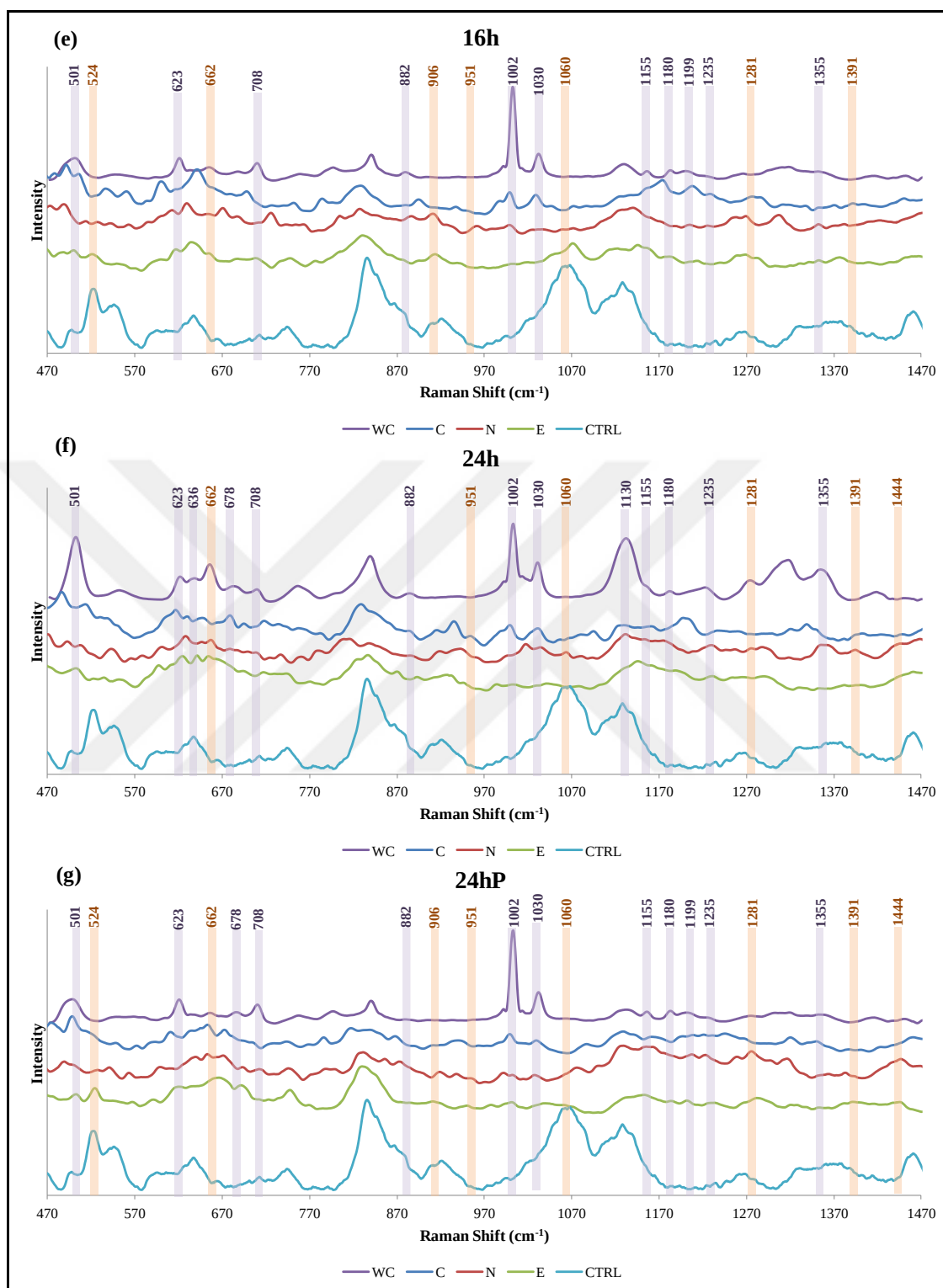


Figure 4.72. Average SERS spectra of HeLa cells (WC), cytosolic fraction (C), nuclear fraction (N) isolated endosomes and homogenization buffer (CTRL) with (a) 15 min, (b) 30 min, (c) 1 h, (d) 4 h, (e) 16 h, (f) 24 h and (g) 2 h pulse 22 h chase AuNP treatment. Spectral bands overlap with the cellular spectra are shown with purple and remaining spectral bands are shown in orange

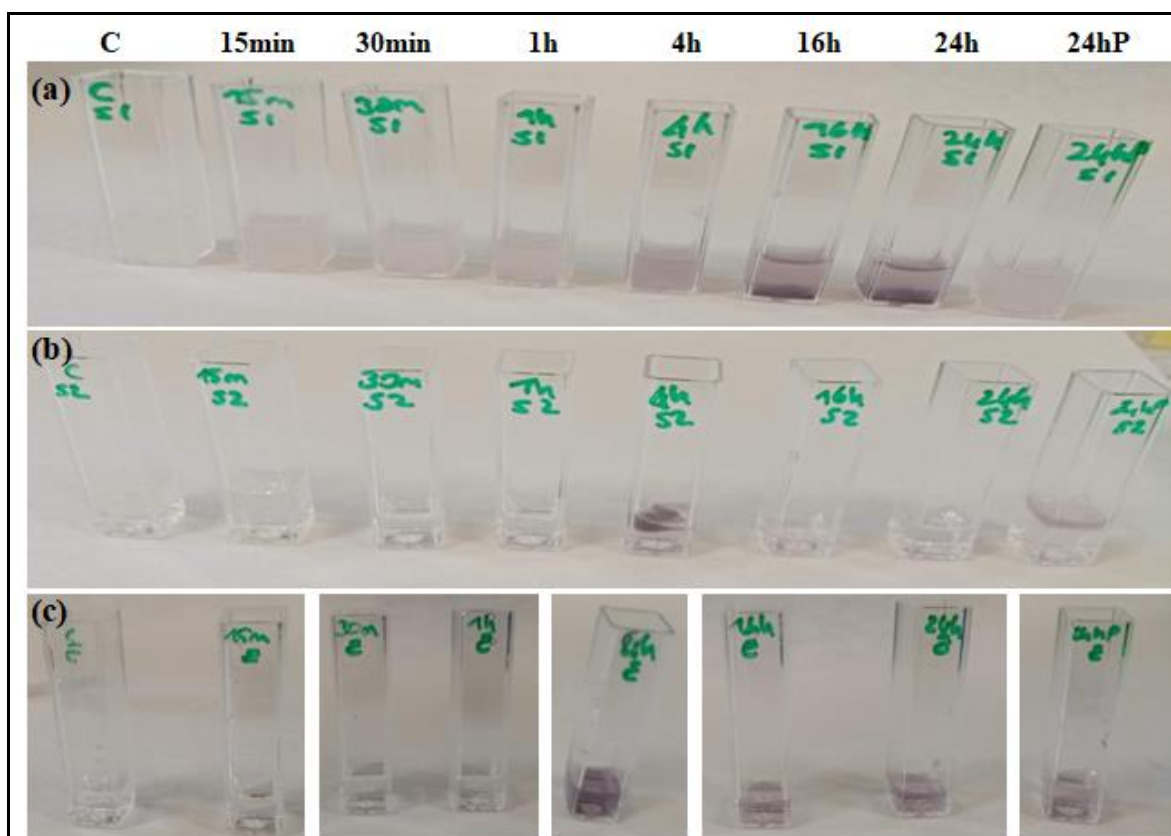


Figure 4.73. Images of the (a) cytosolic fractions, (b) nuclear fractions and (c) isolated endosomes without AuNPs treatment (C) and with 15 min, 30 min, 1 h, 4 h, 16 h, 24 h, 2 h pulse 22 h chase AuNP treatment

After the comparison of the obtained fractions with control groups to observe obtained spectral bands with the isolation, SERS spectra of the fractions could be compared with the intracellular SERS spectra to see how intracellular SERS spectra is composed. Even SERS spectra of cells and fractions were obtained in a different environment; it was found that spectral bands found in the intracellular spectra overlap with different fractions as seen in Figure 4.74. For instance; in the cellular spectra obtained peaks at 501 cm^{-1} (S-S), 623 cm^{-1} (C-C), 636 cm^{-1} (C-S), 708 cm^{-1} (C-S), 1199 cm^{-1} (Trp) were found to overlap with the endosomal fraction. Furthermore, the main question about the origin of the intracellular spectra was also answered with this comparison. Because it was found that intracellular peaks not only overlap with the endosomal fraction, they can also overlap with cytosolic and nuclear fractions. For instance, observed peaks at 678 cm^{-1} (Ring breathing), 882 cm^{-1} (Proteins), 1002 cm^{-1} (Phe), 1030 cm^{-1} (Phe), 1155 cm^{-1} (Proteins) overlap with the

cytosolic fraction and peaks at 1130 cm^{-1} (phospholipid), 1180 cm^{-1} (Tyr, Phe), 1199 cm^{-1} (Trp) and 1355 cm^{-1} (Amide III) was found to overlap with the nuclear fraction.

In the literature, origin of the intracellular spectra is mostly thought as endosomal pathway. Kneipp et al showed that AuNPs are uptaken by endocytosis and during the maturation of the endocytosis, obtained SERS signal changes due to the altered endosomal environment which also affect aggregate formation [311,335]. Moreover, when interaction of different sized AuNPs with the intracellular environment were investigated, obtained cellular spectra was explained by the changes in the endosomal maturation steps which change endosomal environment [388]. While most of the reports in the literature focused on the endosomal environment changes to explain obtained SERS spectra, it was speculated that without any targeting, AuNPs could escape from the endosomes and found in different locations inside the cells [495]. This data show that AuNPs are not residing only inside the endosomes after internalization and obtained intracellular SERS spectra is affected by the surrounding environment of the all AuNPs found in cytosol, nucleus and endosomes. It should be also noted that, pattern of the obtained intracellular spectral does not seem close to the fractional spectra because fractional spectra was obtained by single acquisition by searching SERS signal while intracellular SERS spectra is obtained by directly mapping the cells which includes also Raman signals without any AuNPs enhancement. Thus, when measurements in the cellular map are averaged, it gives different pattern due to not only composed of enhanced SERS signals but also Raman signals without any enhancement.

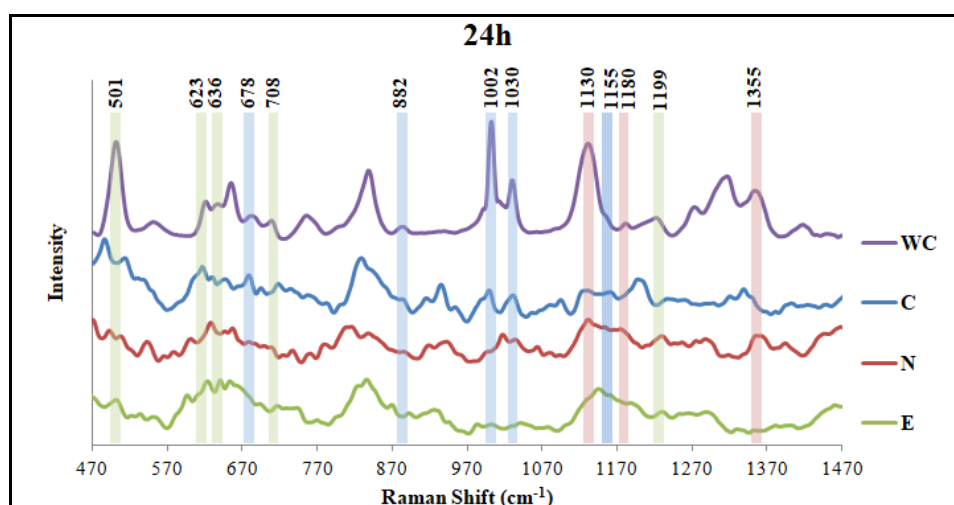


Figure 4.74. Comparison between the cellular SERS spectra (WC) with cytosolic (C), nuclear (N) and endosomal (E) fractions after isolation

4.4.5. Statistical Analysis

Statistical analysis of obtained SERS spectra was examined by LDA. LDA plot of the isolated endosomes can be seen in Figure 4.75. As seen; control group without AuNPs is separated from the remaining AuNPs filled endosome groups and when different time points were compared, although distinct separation between the time points not achieved, each group was found in a different spot as slightly separated in the LDA plot which shows each time point has own characteristic SERS spectra

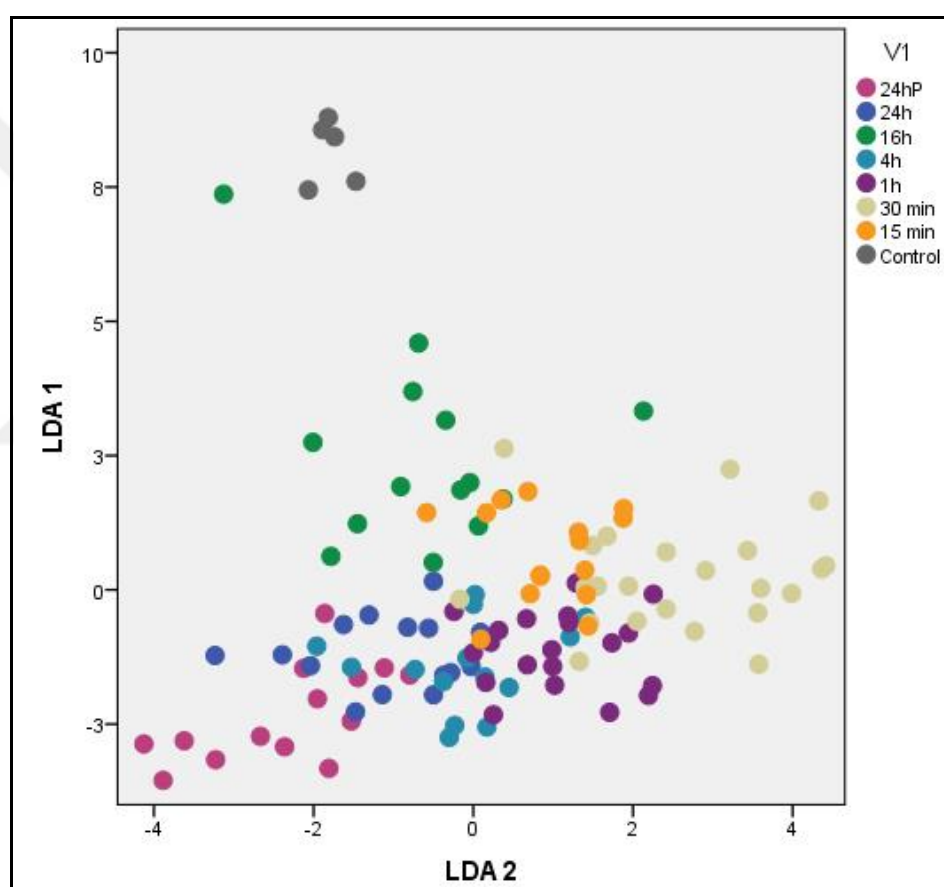
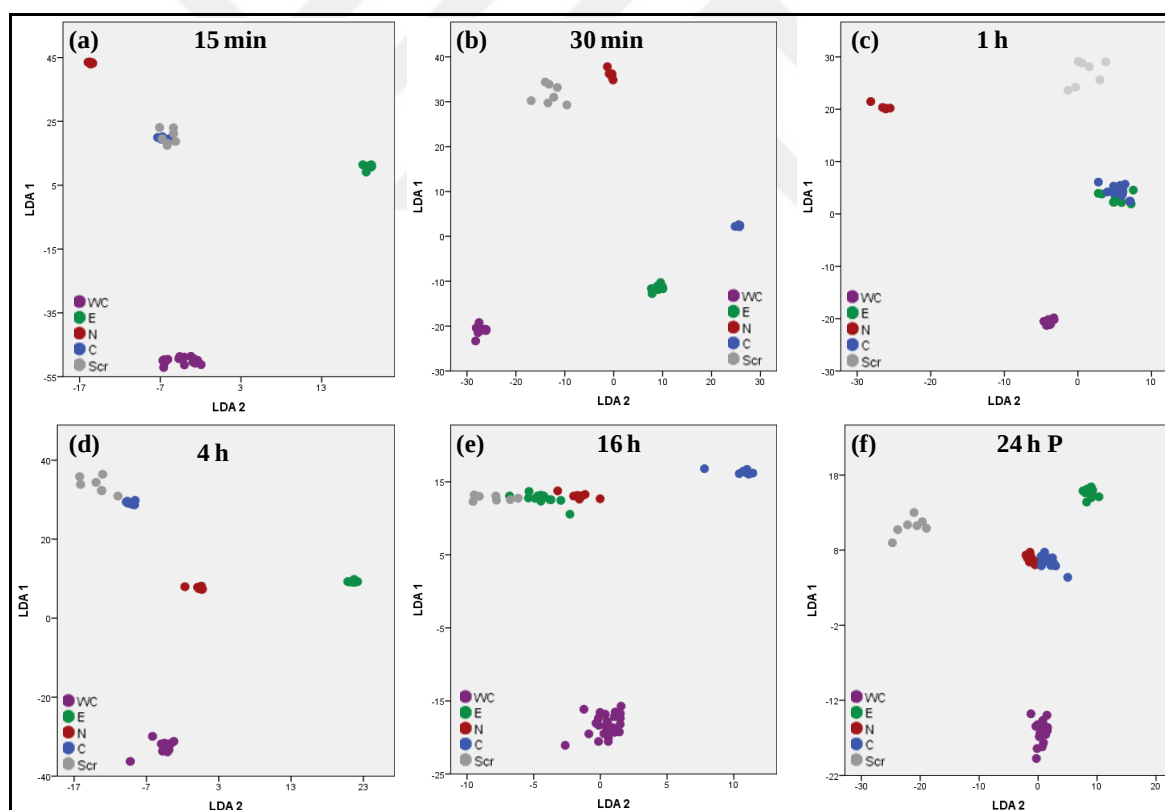


Figure 4.75. LDA plots of isolated endosomes without AuNPs treatment (Control) and with 15 min, 30 min, 1 h, 4 h, 16 h, 24 h, 2 h pulse 22 h chase AuNP treatment

When obtained fractions were compared and analyzed by LDA plots as seen in Figure 4.76., time dependent changes were observed. At 15 min treatment, SERS spectra of the cytosolic fraction was overlapped with control group (Scr) which means obtained spectral information is coming from the sucrose in the homogenization buffer and there was not any SERS signal obtained from AuNPs inside the cytosol of the cell. On the other hand;

SERS spectra of whole cell, nuclear and endosomal fractions were well separated which shows there are AuNPs in the endosomal and nuclear fractions and obtained SERS spectra can be distinguished from other fractions easily. For 30 min treatment, each fraction was found separated which shows AuNPs in the cytosolic, nuclear and endosomal fractions and also inside the cells provided distinct SERS spectra. With 1 h treatment, similar with 15 min treatment, cytosolic fraction was overlapped with control group and remaining fractions and cellular spectra was found as separated with different spectral patterns. After 1 h treatment, spectral bands were observed clearly and in LDA plots, each fraction and cellular spectra was also found as separated. Obtained results showed that, after 1 h treatment, AuNPs escape from the endosomes to the cytosol or the nucleus can be easily tracked by SERS and analyzed by LDA.



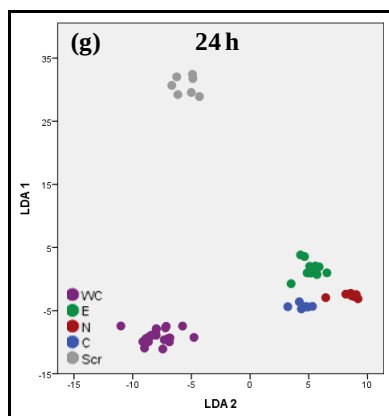


Figure 4.76. LDA plots of HeLa cells (WC), cytosolic fraction (C), nuclear fraction (N) isolated endosomes and homogenization buffer (CTRL) with (a) 15 min, (b) 30 min, (c) 1 h, (d) 4 h, (e) 16 h, (f) 24 h and (g) 2 h pulse 22 h chase AuNP treatment

4.5. MACROPHAGE ACTIVATION STUDIES

Macrophages and their responses have vital role in immunity and immune responses. Their phagocytosis ability makes them among the most important defense mechanism against various microbial and viral infections. Defense by macrophages is obtained by the secretion of pro-inflammatory cytokines such as TNF- α , IL-1B, IL-6 and also secretion of nitrite. In this study, the macrophage activation by LPS and response of activated macrophages to the different concentrations of AuNPs were investigated.

4.5.5. Cytotoxicity of AuNPs on Macrophages

Cell proliferation results of RAW 264.7 macrophages treated with increasing concentrations of AuNPs are shown in Figure 4.77. As seen, AuNPs cause dose-dependent cytotoxicity on macrophages on the other hand 25 percent AuNPs that is used for the control group in SERS experiments were found non-toxic to macrophage cells. At the highest dose, approximately 95 percent of the population was died due to AuNPs cytotoxicity.

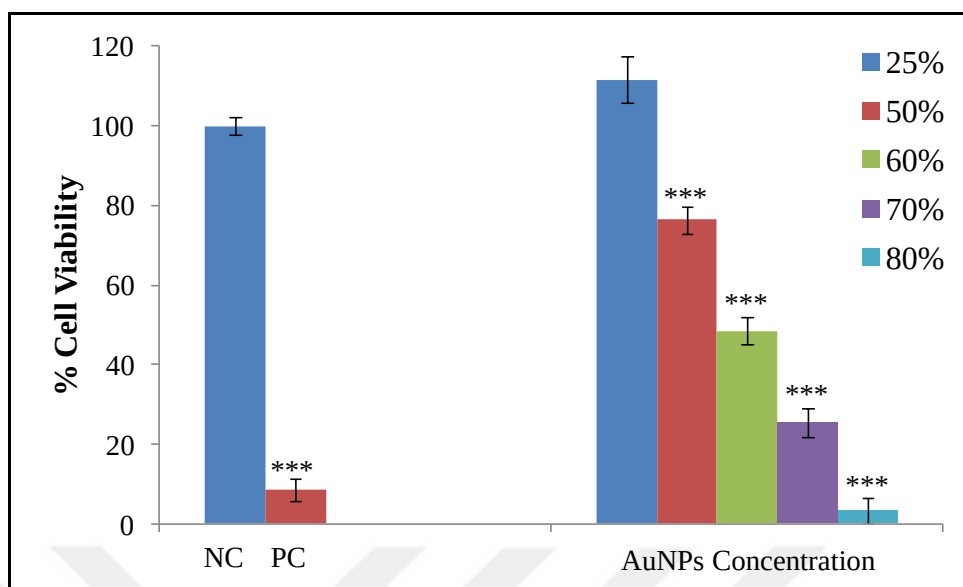


Figure 4.77. WST-8 cell proliferation results of RAW 264.7 treated with AuNPs. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$. PC: Positive control (10 percent DMSO was used as positive control), NC: Negative control

4.5.6. Apoptosis/Necrosis Assay

Apoptosis/Necrosis assay results of macrophages treated with increasing concentrations of AuNPs are shown in Figure 4.78. and it can be observed that AuNPs cause necrosis in both 25 and 80 percent AuNPs treatment on the other hand necrosis were also found in the control cells. Increase in the necrosis can be correlated with the cytotoxicity results in Figure 4.77. which shows very low cell viability at the 80 percent AuNP treatment. Thus, high concentration of the AuNPs could cause necrosis on macrophages. Moreover, these results should be considered in the evaluation of the SERS spectra and necrosis related changes should be pointed.

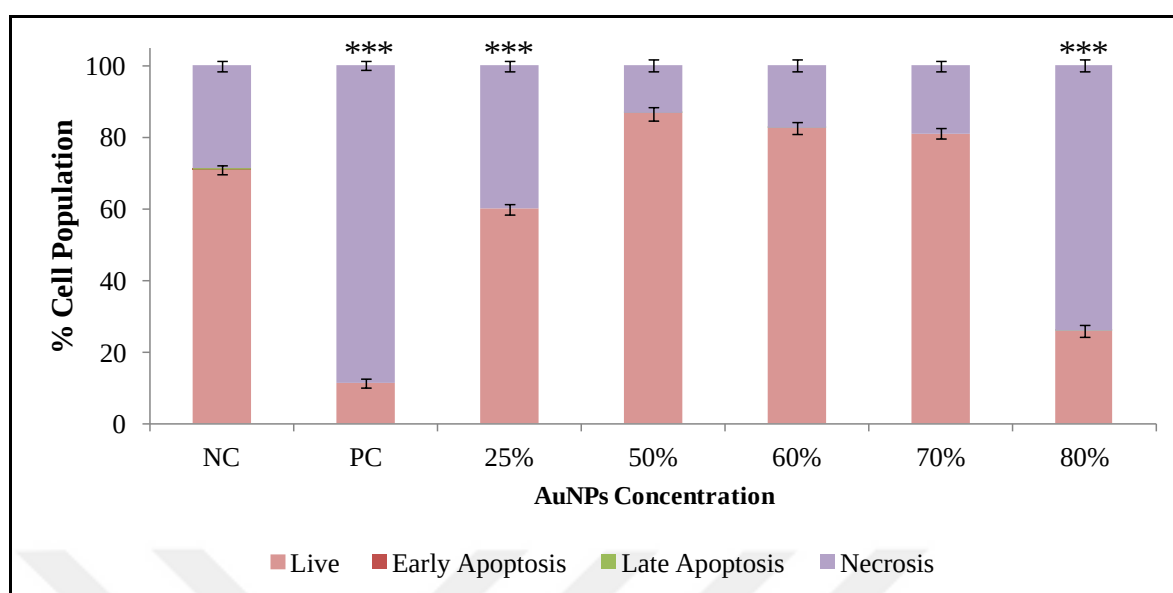


Figure 4.78. Apoptosis/necrosis assay results of RAW 264.7 treated with AuNPs. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$. PC: Positive control (10 percent DMSO was used as positive control), NC: Negative control

4.5.7. Cellular Uptake of AuNPs

Uptake of the AuNPs was investigated in the presence and absence of the LPS as shown in Figure 4.79. with the use of SSC shift in flow cytometry. As seen, even only LPS caused SSC shift as a result of higher granulation after internalization of the LPS when compared with the untreated control group. This is probably caused by the increase in vacuoles within the activated cells [496].

Furthermore, AuNPs uptake was observed easily from the SSC shift and presence of the LPS (LPS+Au) caused significant increase in the SSC shift compared with the only LPS treatment. It could be caused by the co-treatment of LPS and AuNPs and their ability to increase SSC shift by themselves and when they come together both LPS and AuNPs increased SSC shift which is resulted as higher SSC shift when compared with only LPS treatment. Besides, when AuNPs were added to the cell culture after the LPS treatment (LPS-Au), significant increase was observed when compared with only AuNPs treatment. This observation could be the result of induced phagocytosis ability of activated macrophages. Because it is known that when macrophages were activated by LPS, they

can internalize more AuNPs than not-activated macrophages [496,497]. Thus, when macrophages were activated and then treated with AuNPs, this could cause higher uptake rate of AuNPs to phagocytose.

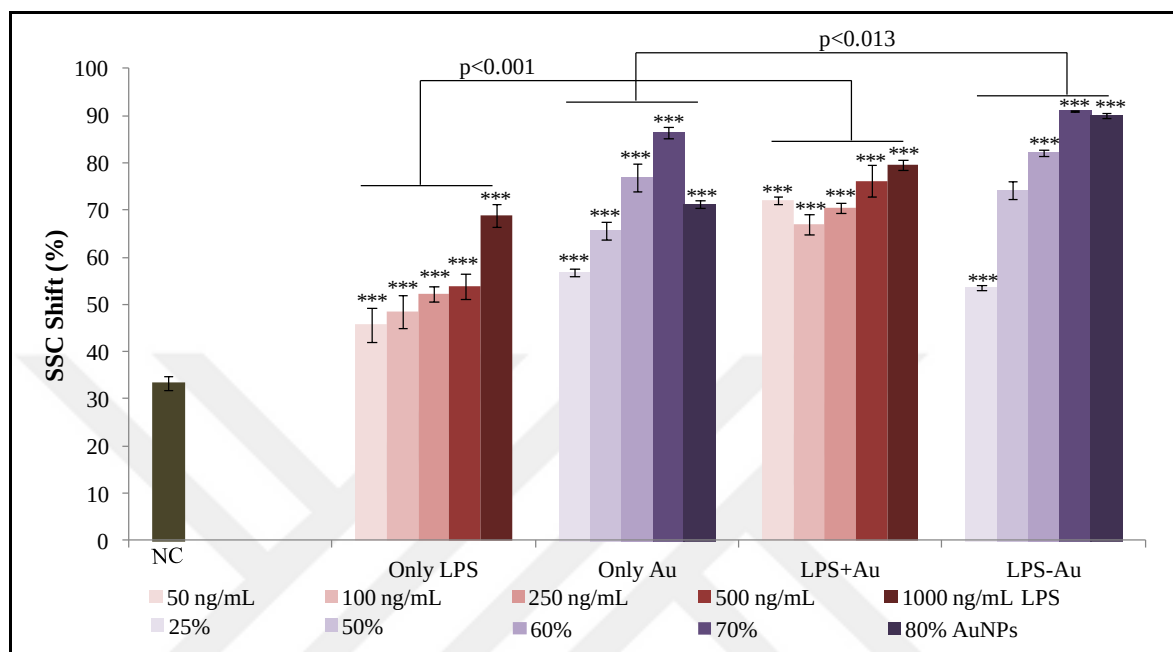


Figure 4.79. Flow cytometry analysis of RAW 264.7 cells exposure to LPS, AuNPs, LPS and AuNPs simultaneously, and LPS and then AuNPs. Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$. NC: Negative control

4.5.8. Detection of Nitrite

Nitrite formation from the nitric oxide metabolism after activation of macrophages was detected for only LPS, only AuNPs, LPS and AuNPs (LPS+Au), LPS then AuNPs (LPS-Au) treatments. Detection was achieved from the supernatant of the cells using Griess reagent. Obtained results were shown in Figure 4.80. As seen, activation by LPS cause highest nitrite amount increase as expected due to the being a well-known biomacromolecule for the activation of macrophages into M1 type [261,498,499].

Moreover, only AuNPs could also cause nitrite increase by itself as a dose dependent manner. It is known that AuNPs could cause immunological responses and when interaction with the macrophages were taken into account, AuNPs could cause differential

effects on macrophage based on their properties such as size, surface charge and chemistry [293]. In this study, it was found that 50 nm AuNPs could cause nitrite level increase. This is an expected result, because AuNPs could cause activation of macrophages and induce secretion of pro-inflammatory cytokines and also nitrite [500]. As a result, it can be said that 50 nm AuNPs caused activation of macrophages.

On the other hand, when LPS activation was achieved with AuNPs treatment simultaneously (LPS+AuNPs), significantly lower nitrite level increase was achieved than only LPS treatment and this situation could be caused by the inhibitory effect of the AuNPs on the LPS-induced activation of macrophages. In the literature it was shown that AuNPs could reduce nitrite level by inhibiting inducible nitric oxide synthase (iNOS) expression at the transcription level and can also inhibit IL-1 β -induced activation and block phosphorylation of MAPKs and Akt, suppress IK β - α degradation which are important steps of the expression of inflammatory genes, iNOS and release of proinflammatory cytokines. As a result, AuNPs block iNOS expression by inhibiting degradation of IK β - α through the inhibition of PI3K/Akt pathways [288,496,501,502].

When AuNPs were used after the LPS activation (LPS-Au) it was seen that even there is a significant nitrite level increase, it provided lowest increase when compared with LPS treatment with or without AuNPs. This is an expected result because before AuNPs were added to the cell culture media, the media including released nitrite from the LPS-induced activation is removed, nitrite release due to LPS-induction is lost and fresh media including AuNPs were added and even after 24 h from the activation (total 48 h treatment), nitrite increase was observed but it was not much like the 24 h treatment of LPS in the absence or presence of AuNPs. On the other hand, AuNPs could also affect and increase the nitrite level after LPS-induced activation because it can also cause nitrite increase by itself without any LPS induction as mentioned above.

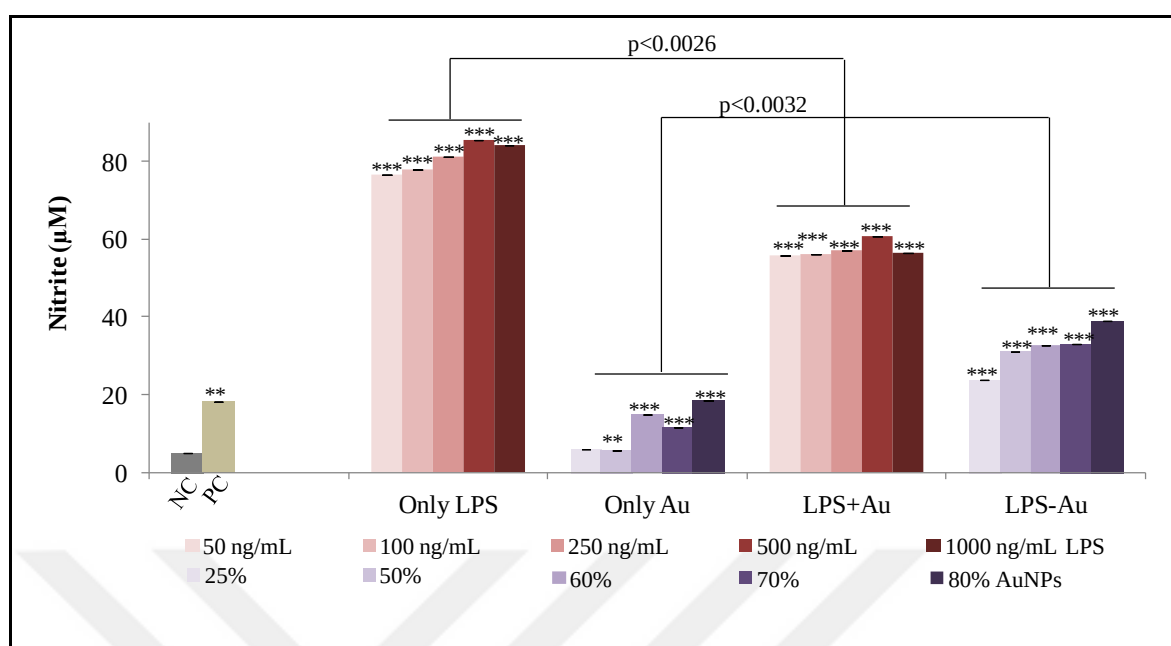


Figure 4.80. Nitric oxide Levels of RAW 264.7 treated with LPS (only LPS), AuNPs (Only Au), LPS and AuNPs (LPS+Au), LPS then AuNPs (LPS-Au). Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$. PC: Positive control (100 µM L-NAME was used as positive control), NC: Negative control

4.5.9. ROS Assay

ROS assay results of macrophages treated with only LPS, only AuNPs, LPS and AuNPs, LPS then AuNPs are shown in Figure 4.81. As seen, obtained results were similar with nitrite increase which is expected. With LPS treatment, highest ROS level is achieved and AuNPs treatment was also caused ROS increase without any activation of macrophages. On the other hand, when AuNPs were used with LPS, ROS level increased but it was lower than only LPS treatment. Moreover, when macrophages were exposed to AuNPs after LPS treatment, significant ROS increase was achieved but it was the lowest increase when compared with all treatments.

ROS are physiologically significant species that can not only cause oxidative stress and cell death at high concentrations but also cellular functions such as cellular signaling, gene expression, protein translation, and protein interactions at distinct levels [503]. From the

immunological point of view, ROS could be used for the polarization of immune cells, phagocytosis process, control of the cell death [504].

For the increase of the ROS at the LPS-induced macrophages, it should be caused by the macrophage activation process by LPS. It is known that M1 macrophages produce nitrite and ROS when they are contact with pathogen. They use NADPH and NADPH oxidase through pentose phosphate pathway for the production of ROS [505] and when macrophages were stimulated by LPS, it promotes recognition by TLRs and interaction of LPS with receptors lead the production of ROS and genes alterations. This induction is Nox-dependent and leads TNF- α production [506]. On the other hand, ROS can be used as a secondary messenger in signal transduction in LPS induced activation and provide regulation of downstream pathways including MAPK and NF- κ B [507]. Thus, LPS-induced activation of macrophages causes ROS production as seen in Figure 4.81. When LPS treatments were compared in the absence (Only LPS) and presence of AuNPs (LPS+Au), it can be seen that presence of AuNPs cause diminish in the ROS production. This should be the result of the inhibition of macrophage activation by AuNPs as seen in nitrite formation results. Thus, with the inhibition of activation by AuNPs, lower ROS is achieved when compared with LPS treatment in the absence of AuNPs.

Besides the role in activation, ROS also have roles in the phagocytosis and cell death for macrophages. Nox2-dependent extracellular ROS is essential for phagocytosis and mitochondrial ROS (mROS) could also enhance phagocytosis though β -oxidation of fatty acids which is associated with the mROS production and bactericidal activity of macrophages [508]. On the other hand, ROS increase can also cause damage on the cells due to the disrupted redox balance. Cell death induction by ROS could be caused by the Nox2 activity in phagosomes [509] and this situation was observed in the AuNPs treatments which caused cell viability decrease in dose dependent manner and with the increased cell death by AuNPs, ROS increase was observed due to the interaction between cell death and ROS. For the comparison between AuNPs treatment without LPS (Only Au) and after LPS (LPS-Au), higher ROS production was observed at LPS-Au treatment and lower concentrations of AuNPs and only Au treatment provided higher ROS production for the higher concentrations. This alteration was probably caused by the cell death coming from AuNPs. For the lower concentrations of AuNPs, ROS production of LPS-Au treated cells was more than only AuNPs treated cells due to the LPS treatment caused ROS

production through activation process and there was not any ROS production at lower doses of AuNPs because there was not any activation agent and also cytotoxicity coming from AuNPs. On the other hand, at higher doses of AuNPs, massive part of the macrophage population up to 95 percent died as in Figure 4.77. which is resulted as higher ROS production than the activation process by LPS.

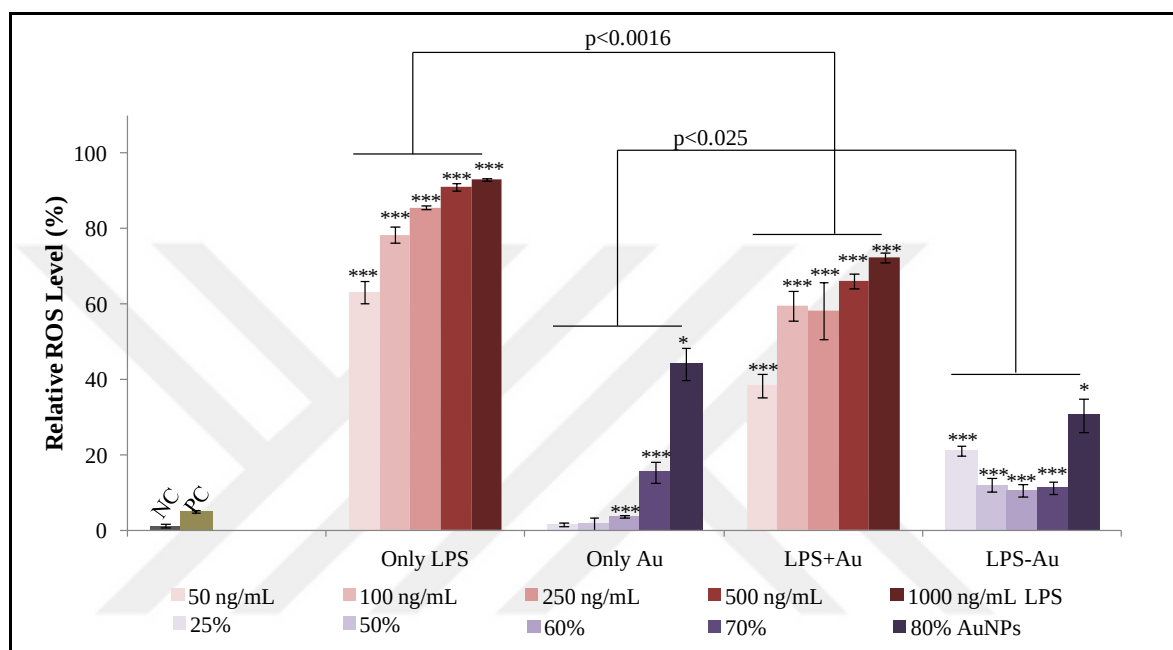


Figure 4.81. ROS assay results of RAW 264.7 cells treated with LPS (only LPS), AuNPs (Only Au), LPS and AuNPs (LPS+Au), LPS then AuNPs (LPS-Au)

4.5.10. SERS Measurements

Activation of macrophages by LPS was investigated by SERS as seen in Figure 4.82.-4.85. For the activation of macrophages, LPS was used, and activation mechanism was investigated via co-treatment of LPS with AuNPs (LPS+Au). On the other hand, response of the M1 macrophages was also examined via LPS treatments and then AuNPs treatment (LPS-Au). For this type of investigation, different concentrations of AuNPs were used to see the response of macrophages. As control experiment, AuNPs dose dependent SERS experiment were also investigated as seen in Figure 4.82. As shown, dramatic changes were not observed with increasing concentrations of AuNPs although high concentrations of AuNPs found cytotoxic and cause necrosis. Cytotoxic effects of the AuNPs could not

observe and this was probably caused by the removal of dead cells during elimination of cell culture media and washing steps before SERS measurement. When cells were treated with 80 percent AuNPs, only approximately 5 percent of the cell population was alive, and in the apoptosis/necrosis assay, dead cells were also collected from cell culture media. However, to remove the AuNPs in the cell culture media which includes dead cells have to be removed to eliminate false SERS measurements coming from AuNPs in the cell culture media and thus, not significant changes were observed in the spectra. On the other hand, when obtained changes were considered, peak intensities originating from proteins (636, 1272-1352 cm^{-1}), amino acids (1030, 755, 838 cm^{-1}) and nucleic acids (1418 cm^{-1}) observed to changed which could be resulted from the necrosis of the cells leading cellular disruption, protein degradation and denaturation, DNA fragmentation [353,355].

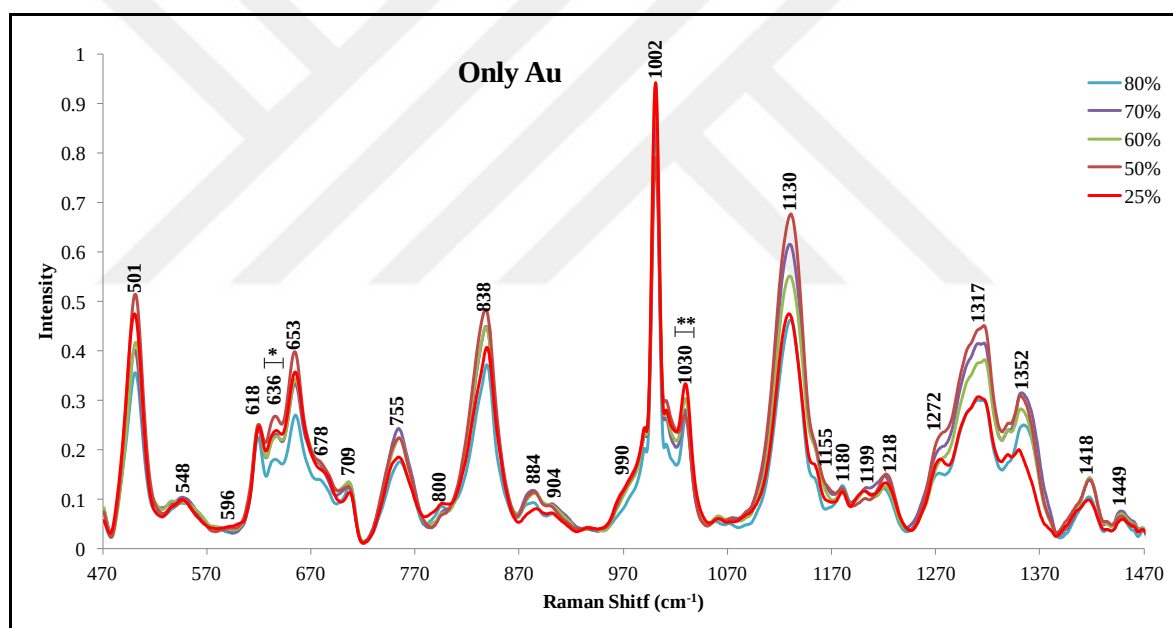


Figure 4.82. Average SERS spectra of RAW cells treated with increasing concentrations of AuNPs (Only Au). Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$ with the comparison of 80 percent AuNPs and 25 percent AuNPs treated groups

For the investigation of macrophage activation mechanism by SERS, cells were treated with LPS and AuNPs simultaneously and obtained cellular SERS spectra are shown in Figure 4.83. As seen, when LPS and AuNPs were used together, the peak intensities originating from proteins (501, 636, 653, 709, 755, 838, 1030, 1272-1352 cm^{-1}),

phospholipid (1130 cm^{-1}) and phosphatidylinositol (596 cm^{-1}) were found to be changed significantly which is caused by the M1 type activation mechanisms induced by increasing concentration of LPS treatment.

LPS is the most potent microbial initiator of inflammation with a complex glycolipid including hydrophobic lipid A domain and hydrophilic polysaccharide found on the outer membrane of Gram-negative bacteria [510,511]. LPS induced activation process produces proinflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukins (IL) such as IL-1, IL-6, IL-8 and IL-12 [510,511] and secrete inflammatory cytokines and mediators including platelet-activating factor, transcription factors such as nuclear factor κ B (NF- κ B) and activator protein-1 (AP-1) prostaglandins, enzymes, and free radicals such as nitric oxide [512-514].

When macrophages were stimulated with LPS, it binds first to a serum LPS binding protein (LBP) and LBP transfers LPS monomer to membrane bound CD14 and then myeloids differentiation protein 2 (MD-2)/TLR4 complex which initiates LPS response [515-517]. After binding of LPS to MD-2, dimerization of MD-2/TLR4 complex is induced and this induction initiates the myeloid differentiation factor (MyD88)-dependent and -independent pathways [518].

With the activation of MD-2/TLR4 complex, Toll-interleukin-1 receptor (TIR) domain of TLR4 refolds and recruits 4 adaptor molecules TIRAP, MyD88, TRAM and TRIF. With recruitment of TIRAP and MyD88, MyD88 dependent pathway is activated through I κ B kinase (IKK) which provides the activation of nuclear factor (NF)- κ B and expression of pro-inflammatory cytokines such as TNF, IL-1 β , IL-6, IL-8, IL-12, IL-23 [519,520]. With the recruitment of TRAM and TRIF, MyD88-independent pathway is activated and this activation is resulted as the activation of interferon regulatory factor (IRF3) which is used for the expression of type I IFNs including IFN- β [521,522].

This mechanism of activation could be observed by SERS as the intensity changes of certain peaks, because even it is known that AuNPs were internalized via endocytosis by cells [207,209,210], when macrophages were concerned, they prefer phagocytosis instead of endocytosis [523-525]. This will lead LPS and AuNPs take the same route in the cell and provide the SERS based observation of environmental changes near or on the AuNPs which are coming from activation mechanism.

In the SERS spectra, it can be seen that the peak intensities originating from proteins ((501, 636, 653, 709, 755, 838, 1030, 1272-1352 cm^{-1}) were changed as increase and also decrease. This alteration was caused by the progress of activation mechanisms and proteins involved in different steps such as G-proteins [526] and kinases such as mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase (ERK) 1 and ERK2 [527], p38 MAPK [528], c-Jun N terminal kinases (JNK) [529], protein kinase A [530], protein kinase C [531], and Src-related kinases [532]. Moreover, before the activation of signaling pathways, LPS interacts not only receptors but also proteins such as MD-2 and CD14, on the other hand when signaling pathways were activated by the interaction, many transcription factors were included, regulatory proteins were recruited. As a result, pro-inflammatory cytokines were produced; inflammatory cytokines and mediators were released by macrophage. All of these changes around AuNPs in the cytosol and phagosomes caused spectral changes in the protein related peaks of SERS spectra.

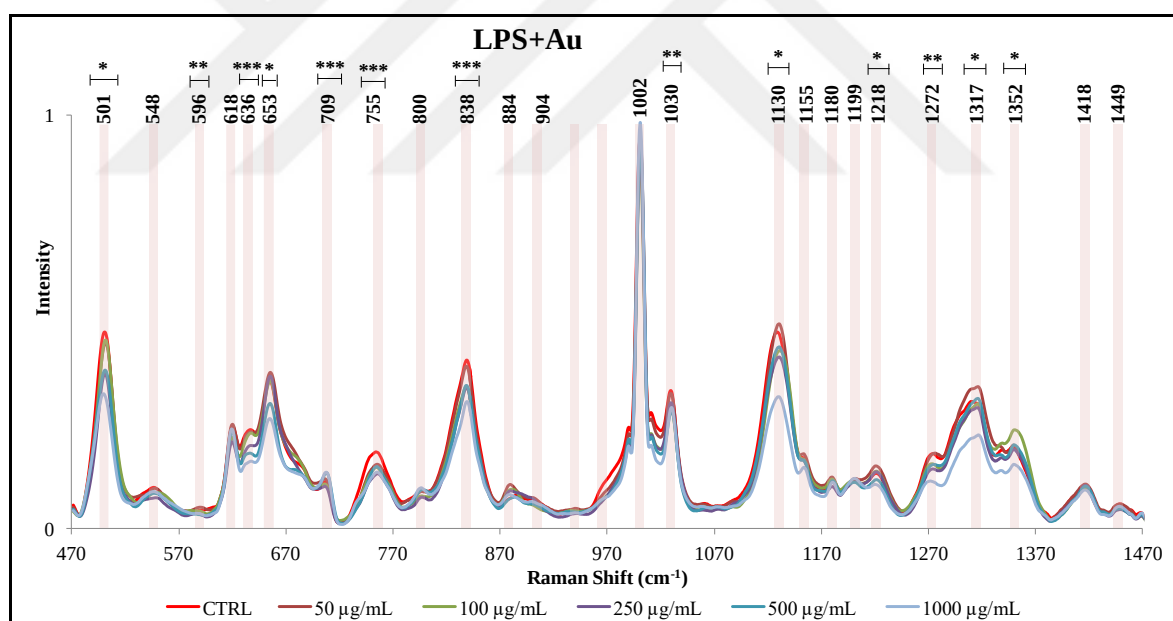


Figure 4.83. Average SERS spectra of RAW cells treated AuNPs (25 percent v/v) and increasing concentrations of LPS simultaneously (LPS+Au). Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$ with the comparison of 1000 $\mu\text{g/mL}$ LPS and control group

Besides the investigation of activation mechanism, response of the M1 macrophages against AuNPs was also examined by SERS as seen in Figure 4. 85. When compared with the obtained SERS spectra, most dramatic changes were observed with LPS-AuNPs treatment. When cells were co-treated with LPS and AuNPs, LPS and AuNPs take the same route and found in the steps of macrophage activation. On the other hand, when LPS was given first without AuNPs, macrophages activated by LPS but then LPS including cell culture media is removed and different concentrations of AuNPs were encountered in the cell culture media. This will cause a response of M1 macrophage against AuNPs. In the obtained SERS spectra, with the increase of AuNPs concentration, dramatic changes were observed in most of the peaks originating from not only proteins but also membrane structures including cholesterol and phospholipid and also nucleic acids.

When AuNPs were given to the M1 macrophages, they will be phagocytosed by macrophages. In the early steps of phagocytosis, particles were recognized by receptors as seen in Figure 4.84. In the case of AuNPs, different pattern recognition receptors (PRRs) were reported to use for AuNPs recognition. Bastus et al showed that 15 nm AuNPs were recognized by TLR4 efficiently [290,291] while cytosine-phosphate-guanosine (CpG) oligodeoxynucleotide conjugated 15 and 30 nm AuNPs and also PEGylated AuNPs with different sizes (4, 11, 19, 35 and 45 nm) were recognized by TLR9 [524,533]. Furthermore, scavenger receptors were also found to have vital role in the AuNPs uptake [364,534,535]. Different types of receptors could be included in the internalization process due to the fact that receptor cooperation is required for the initiation of phagocytosis [102].

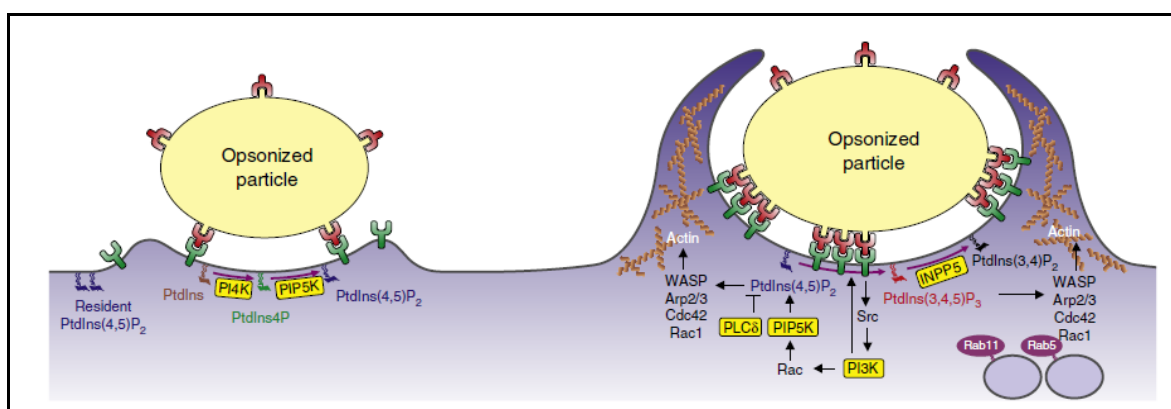


Figure 4.84. Schematic representation of early steps of phagocytosis [433]

After phagocytic receptors recognize target particle and aggregate to initiate signaling pathways, actin cytoskeleton is regulated for the membrane protrusions and formation of pseudopodia. As the first step of actin remodeling, membrane-associated cortical cytoskeleton is disrupted, then F-actin polymerization is initiated with actin filaments nucleation and pseudopodia is extended and phagocytic cup is formed for the internalization of the target. As a last, actin depolymerization occurs for the formation of phagosome [120].

During these steps, AuNPs were covered by remodeled plasma membrane and actin is used for the remodeling of the membrane [102]. Interaction with the receptors and their mobility, membrane remodeling and actin involvement during the phagosome formation could lead the intensity changes of the peaks originating from lipid structures such as phospholipids (1130 cm^{-1}) and cholesterol (548 cm^{-1}).

During the activation of phagocytosis, series of signaling events were triggered including Fc γ signaling [116], lipid signaling [117], small GTPase signaling [118], and complement receptor signaling [119]. Among the pathways, lipid signaling is an important mechanism in the phagocytosis progress. Phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) is found in the inner leaflet of the plasma membrane of phagocytes as large amounts. In the early phases of phagocytosis concentration of the PI(4,5)P₂ increases for the formation of phagocytic cup [117]. Then PI(4,5)P₂ disappears quickly to allow target particle uptake. Disappearance of the PI(4,5)P₂ is carried out not only actin disassembly but also different molecules including phospholipase C (PLC) γ , diacylglycerol (DAG). PI(4,5)P₂ is converted into PI(3,4,5)P₃ by PI3K which is recruited by Syk [536] or adaptor proteins such as Gab2 [537,538] at the phagocytic cup. These dramatic changes in the lipid signaling will lead changes in not only phospholipid correlated peak intensities but also peaks originating from phosphotyidylinositol (596 cm^{-1}).

As another signaling, small GTPases are used for the regulation of actin cytoskeleton and actin cytoskeleton has a vital role for phagocytosis as used in phagocytic cup and phagosome formation, movement and fusion with lysosomes. Small GTPases are used as molecular switches as GTP bound active and GDP bound inactive states. Guanine nucleotide exchange factors (GEFs) catalyze release of GDP and replacement of GTP for the activation and then GTP is hydrolyzed to GDP with the help of GTPase-activating

proteins (GAPs). After these steps, GTPases Rac and Cdc42 are activated and recruited to the phagocytic site and lead phagocytosis [118]. While Cdc42 is activated in the early steps of the phagocytosis, Rac2 is activated later [539]. They are used for the pseudopodia extension by activating nucleation-promoting factors WASp (Wiskott-Aldrich Syndrome protein) and Scar/WAVE [540]. Scar and WASp activate Arp2/3 complex for actin polymerization [541]. When enhanced GTPase activities were taken into account, increased peak intensities could be observed at the peaks originating from the guanine ($666, 678 \text{ cm}^{-1}$) [367,542].

After the formation of pseudopods, phagocytic cup and lastly phagosome, it matures and fuse with lysosome to form phagolysosomes. In the maturation process, several steps are involved. In the formation of early phagosomes, small GTPase Rab5 is used for the membrane fusion [122] and recruited EEA1 to provide phagosome-early endosome fusion [123]. For the formation of PI3P, class III PI-3K human vacuolar protein-sorting 34 (hVPS34) is also recruited to the site [124]. After the formation of PI3P, maturation is occurred and proteins of early phagocytosis are recycled back and proteins for the maturation are recruited to the site such as Rab7, Rab-interacting lysosomal protein (RILP) [125]. Then lumen is acidified, ILVs are formed, phagosomes fused with lysosome which requires soluble NSF attachment protein receptor (SNARE) and vesicle-associated membrane protein (VAMP) 7 and VAMP8 [126], lysosomal-associated membrane proteins (LAMPs) and luminal proteases [127]. As seen, maturation process of phagocytosis requires recruitment of various proteins to the phagocytic site and then disassembling from the site and then again recruitment of new proteins for maturation. These type of alterations in the protein profile near or on the phagosomes are observed in the SERS spectra as the intensity changes of the peaks originating from proteins such as 618, 636, 653, 755, 838, 882, 1002, 1272, 1352 cm^{-1} .

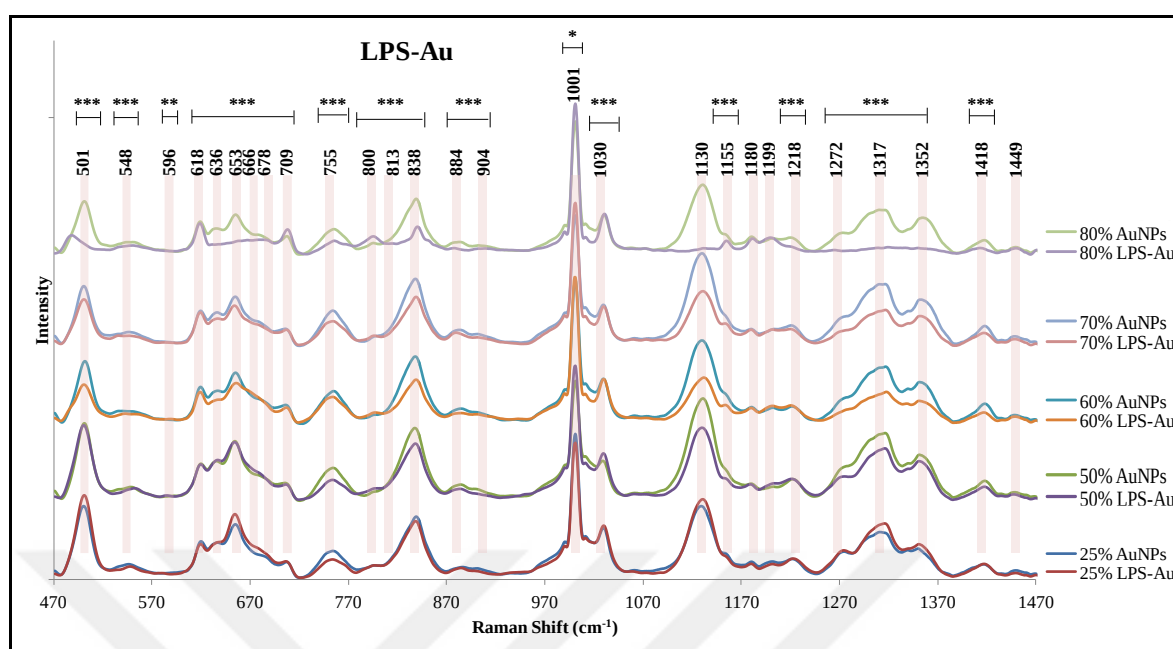


Figure 4.85. Average SERS spectra of RAW cells treated increasing concentrations of AuNPs after treated with 1000 µg/mL LPS (LPS-Au). Statistically significant changes were calculated by two-paired Student's t test, and marked with stars, * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$ with the comparison of and 80 percent AuNPs and control group

4.5.11. Statistical Analysis

For the observation of the variation of the SERS spectra, PCA and LDA was applied to the obtained average cellular SERS spectra after LPS and or AuNPs treatment and obtained LDA plots can be seen in Figure 4.86. From the obtained LDA plots, better separation was achieved with LPS+Au and LPS-Au treatments when compared with only AuNPs treatment as similar to the obtained spectral changes. SERS spectra were also not changed significantly with AuNPs treatments and this is reflected in the LDA plots as well. On the other hand, when LPS+Au and LPS-Au treatments were compared with the control group (25 percent AuNPs treated group), treatments were separated from the control. Moreover, p values for the selected Raman shift in SERS spectra are given as Table 4.17.-4.19.

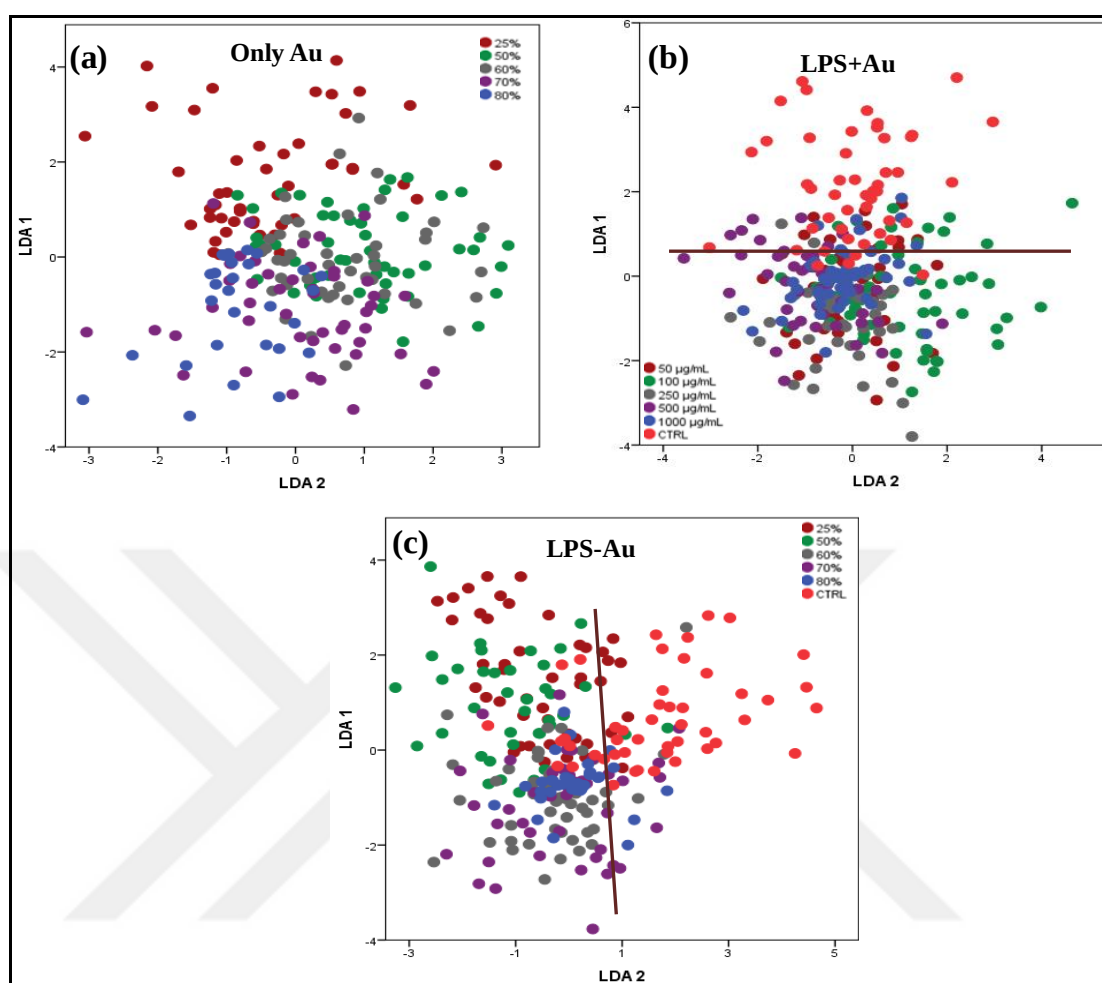


Figure 4.86. LDA Plots of RAW 264.7 cells treated with (a) only AuNPs, (b) LPS and AuNPs simultaneously (LPS+Au) and (c) LPS and then AuNPs (LPS-Au)

When obtained p-values examined as seen in Table 4.17.-4.19., only Au treatment provided different significance when AuNPs concentration changes. It should be the result of comparing each concentration with 25 AuNPs. With the increase in the AuNPs amount, uptake rate changes and with altered uptake rate interaction of AuNPs could change with cell and endosomes and it can be reflected as significant peak intensity changes in the SERS spectra.

Table 4.17. Total p values for the SERS spectrum obtained from RAW 264.7 cells treated with only AuNPs. Red cells show p value < 0.05, yellow highlight shows p value < 0.01, and green highlight shows p value < 0.001

	Only Au	AuNPs Concentration			
		50%	60%	70%	80%
Raman Shift (cm ⁻¹)	1449	0.27470	0.08684	0.01853	0.81474
	1418	0.00018	0.00017	0.00005	0.65692
	1352	0.00016	0.00503	0.00023	0.38853
	1310	0.00171	0.10721	0.01852	0.92696
	1272	0.06485	0.95157	0.37320	0.40560
	1218	0.10650	0.51802	0.27378	0.43676
	1199	0.09230	0.95135	0.47532	0.96338
	1180	0.36150	0.42800	0.20211	0.32951
	1155	0.00518	0.01775	0.01913	0.15253
	1130	0.00348	0.24299	0.04513	0.88529
	1030	0.00006	0.02745	0.00097	0.00142
	1001	0.00018	0.97447	0.03377	0.13147
	990	0.93187	0.54034	0.28807	0.00930
	904	0.10392	0.01932	0.00911	0.96303
	884	0.00014	0.00016	0.00004	0.37050
	838	0.01024	0.20324	0.16482	0.44512
	800	0.00479	0.00473	0.04767	0.62701
	755	0.01288	0.01675	0.00535	0.65356
	709	0.20010	0.30948	0.39601	0.12730
	678	0.22061	0.69208	0.49918	0.05071
	636	0.20803	0.60953	0.77138	0.04466
	618	0.66143	0.51268	0.84664	0.13570
	548	0.82204	0.56821	0.43982	0.48952
	501	0.52651	0.31335	0.23916	0.11770

When macrophages treated with both LPS and AuNPs simultaneously, obtained p-values as seen in Table 4.18. showed that starting from 100 µg/mL LPS, significant changes were observed in the SERS spectra as changed peak intensities and when LPS amount increases, obtained peak intensity changes become more significant. Most significant differences were obtained by highest concentration 100 µg/mL.

Table 4.18. Total p values for the SERS spectrum obtained from RAW 264.7 cells treated with LPS and AuNPs simultaneously at increasing concentrations of LPS. Red cells show p value < 0.05, yellow highlight shows p value < 0.01, and green highlight shows p value < 0.001

	LPS+Au	LPS Concentration				
		50 µg/mL	100 µg/mL	250 µg/mL	500 µg/mL	1000 µg/mL
Raman Shift (cm ⁻¹)	1449	0.91288	0.05101	0.02934	0.06773	0.41199
	1418	0.31104	0.95855	0.60271	0.69869	0.58888
	1352	0.97390	0.25578	0.70321	0.98619	0.03941
	1310	0.50401	0.65556	0.65401	0.90633	0.02250
	1272	0.99658	0.33683	0.19517	0.33144	0.00530
	1218	0.18551	0.66523	0.72679	0.26892	0.03257
	1199	0.50221	0.95408	0.78901	0.47593	0.38335
	1180	0.28091	0.61664	0.12147	0.93907	0.52870
	1155	0.63409	0.86571	0.77511	0.72217	0.06190
	1130	0.80268	0.42740	0.39339	0.58961	0.01003
	1030	0.67031	0.00388	0.06930	0.00013	0.00154
	1001	0.85576	0.12472	0.33291	0.73889	0.08622
	904	0.72087	0.00352	0.94312	0.01802	0.20287
	884	0.21897	0.46044	0.51141	0.42064	0.27923
	838	0.64354	0.02960	0.05747	0.05327	0.00068
	800	0.75089	0.06537	0.02692	0.94878	0.28769
	755	0.02397	0.00991	0.00055	0.01816	0.00039
	709	0.43682	0.42394	0.12275	0.00579	0.00062
	653	0.58579	0.99426	0.78597	0.16611	0.01184
	636	0.83619	0.73849	0.11022	0.00994	0.00028
	618	0.62816	0.00383	0.00056	0.11723	0.31516
	596	0.42994	0.12816	0.00351	0.00246	0.00502
	548	0.82751	0.11621	0.00320	0.07067	0.22456
	501	0.69513	0.76124	0.15079	0.15946	0.01421

Table 4.19. shows p-values of the intracellular SERS spectra obtained from macrophages treated with LPS and then AuNPs. As seen, each treatment concentration provided significant changes in the peak intensities of corresponding spectral bands and using 80 percent AuNPs after LPS treatment provided most significant results which are caused by the increased uptake of AuNPs with increased cell death rate, nitrite and ROS levels.

Table 4.19. Total p values for the SERS spectrum obtained from RAW 264.7 cells treated with LPS and then AuNPs at increasing concentrations of AuNPs. Red cells show p value < 0.05, yellow highlight shows p value < 0.01, and green highlight shows p value < 0.001

	LPS-Au	AuNPs Concentration				
		25%	50%	60%	70%	80%
Raman Shift (cm ⁻¹)	1449	0.00028	0.24394	0.7328	0.62587	0.1177
	1418	0.92366	0.81427	0.09291	0.73621	1.1E-09
	1352	0.31475	0.0159	0.0832	0.75579	1.8E-11
	1310	0.32757	0.50162	0.00559	0.14695	1.2E-11
	1272	0.81902	0.64641	0.00029	0.01308	5E-10
	1218	0.93888	0.19527	0.66782	0.53871	4.5E-09
	1199	0.00685	0.60121	0.17482	0.24519	0.71156
	1180	0.05284	0.8978	0.78773	0.25693	0.44766
	1155	0.13671	0.35215	0.00724	0.76608	9.7E-07
	1030	0.44625	0.39461	0.01736	2.6E-05	1.3E-06
	1001	0.09075	0.10886	0.4244	0.90025	0.02294
	904	0.00229	0.00436	0.99523	0.79596	5.3E-05
	884	0.14628	0.66824	0.89841	0.23586	1.5E-09
	838	0.34216	0.44284	0.00026	0.03622	2.7E-13
	813	0.42125	0.93615	0.00244	0.09429	8.5E-07
	800	0.80827	0.80032	0.35399	0.3796	2.6E-06
	755	6.6E-05	0.00625	0.99585	0.71019	8E-09
	709	0.97682	0.94873	0.91111	0.30498	1.4E-05
	678	0.03078	0.10974	0.28869	0.61448	1.3E-05
	666	0.11284	0.24451	0.49022	0.10361	5.2E-11
	653	0.12112	0.41509	0.01722	0.03783	2.9E-13
	636	0.93476	0.17602	0.00105	0.09136	1.9E-13
	618	0.01809	0.86975	0.00082	0.25906	8.4E-06
	596	0.00957	0.15266	0.30885	0.25167	0.00156
	548	0.02427	0.07872	0.0004	0.07397	8.6E-05
	501	0.31653	0.72646	0.00062	0.01923	6E-11

5. CONCLUSION AND FUTURE PERSPECTIVES

For understanding interaction of NMs with living systems, SERS offers a unique opportunity to obtain valuable information from living single cells without any disruption and labeling. In this thesis, it is aimed to understand the origin of the intracellular SERS spectra and the factors that affect obtained spectra. Under this main purpose, protein corona formation is monitored to understand how SERS spectra evolve during protein corona formation over time in different cell culture media. Further, it is aimed to understand whether SERS spectral data acquired from endolysosomes, where AuNPs mostly reside, as a result of endocytosis in living single cells can answer the question of whether SERS can discriminate endocytosis pathways of AuNPs. Besides, the SERS spectra of isolated endosomes are used to understand the origin of the intracellular SERS spectra by comparing them to the spectra of whole cells. Finally, SERS is evaluated whether it can help to understand the macrophages activation mechanism.

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As the first investigation, formation of the protein corona structure on the AuNPs was investigated. For this purpose, two different cell culture media (DMEM, DMEM:F12 1:1) and two different FBS concentrations (5 and 10 percent) were used. Properties of soft corona (SC) and hard corona (HC) investigated separately with in situ measurements for SC and with the elimination of SC via centrifugation for HC. I found from the

characterization results that for both of the corona types, when AuNPs were added to the cell culture media, sudden corona layer was formed on the AuNPs surface which increased hydrodynamic size, decrease zeta potential value, and provide red shift in the UV/Vis absorbance. Slight time dependent changes are observed which is resulted from the dissociation of soft corona proteins and association of hard corona proteins. From in situ measurements, increased changes in the size, zeta potential values and absorbance spectrum were observed. With the elimination of SC on the AuNPs via centrifugation, obtained changes decreased due to the removal of low affinity SC proteins from the surface. All these changes reflected on the SERS spectrum of PrC structure. When SC is investigated by SERS, first interaction time provided better SERS signal when compared with increasing interaction times. It could be explained by the sudden protein coating on the AuNPs which was also observed in the UV/Vis and DLS results. After the first interaction time, obtained SERS signal intensity decreased and then increased with increasing time. It could be related with the dissociation of SC proteins on the surface which is resulted with the decreased signal, and then association of the new HC proteins which is resulted as the increased signal. When different cell culture media compositions were compared, using different media type provided slight changes in the obtained SERS spectra while using high concentration of FBS provided less SERS signal based on the intensity. The reason behind this situation can be the suppression of the obtained signal coming from the control (FBS included cell culture media). With the increased concentration of FBS, suppression rate could increase which lead less signal intensity. Moreover, affect of the altered PrC structure on the cellular uptake, uptake route and intracellular spectra was also examined. It was observed that using different cell culture media compositions could affect uptake amount of AuNPs and also uptake route. For instance, for the growth of Beas-2b cells, DMEM with 5 percent FBS is used which provides internalization of AuNPs with all three pathways: ClME, CaME and macropinocytosis. However, when FBS rate is increased up to 10 percent, ClME is eliminated from the routes while cell culture media is changes as DMEM:F12, both ClME and CaME is eliminated as the uptake route. With the alterations in the PrC structure, uptake amount and uptake route, obtained intracellular SERS spectra was also change significantly based on the used cell culture compositions. Using different media compositions changes band intensities of all spectra significantly which shows that using different media compositions changes the protein corona structure and this affect uptake

amount and route of AuNPs which directly changes the obtained intracellular SERS spectra. Thus, when intracellular spectra are interpreted, formed PrC structure on the AuNPs should be also considered.

After the investigation of PrC structure, endocytosis pathway choice of AuNPs was examined by SERS. For this purpose, 9 different inhibitors specific for the endocytosis pathways were selected and inhibition conditions were optimized. From the optimization results it was found that use of 2h non-continuous inhibition in the presence of FBS could diminish the toxic effect of inhibitors while still provide uptake inhibition. Then CaME, ClME, macropinocytosis, dynamin dependent endocytosis and receptor mediated endocytosis were inhibited.

As the first pathway, clathrin mediated endocytosis (ClME) was inhibited by Scr and Cpm. It was found that effect of the Scr and Cpm differentiates. Although both Scr and Cpm cause dose dependent cytotoxicity on all cell lines, Scr caused uptake inhibition for Beas-2b, A549 and PNT1A cells while Cpm caused AuNPs uptake inhibition only for PNT1A cells. When obtained cellular SERS spectra in the presence of Scr was investigated, it was found that treatment with Scr provides significant changes in the peak intensities attributed to phospholipids and proteins. When cells were treated with Scr, dispersion of clathrin lattices hindered and ClME is blocked. When ClME is blocked from the initial steps where clathrin is used for the formation of clathrin coated vesicle formation and maturation, pathway cannot be developed for the formation of early endosomes, late endosomes and endolysosomes. Thus, microenvironment where AuNPs reside is changed and these changes reflected on the SERS spectra as significant changes. Furthermore, these changes were related with not only natures of different cell types but also the different protein corona found on the AuNPs in different cell culture medium and also differential uptaken rate of AuNPs by different cell types. It was observed that Scr treatment affected most PNT1A cells. And it was also found that PNT1A cells are the cell type whose uptake of the AuNPs is the least and thus when AuNPs uptake was also inhibited by Scr treatment, it was affected most, and spectral changes were observed dramatically when compared with other cell lines. Cpm was also used for the inhibition of ClME in PNT1A cells which interact with the clathrin and AP2 adaptor complex. With the inhibition of important proteins responsible for the initiation of vesicle scission, vesicle formation, and maturation, pathway is blocked and AuNPs could not use ClME for the internalization, thus spectral

bands changed significantly with the elimination of one of the most preferred pathways for AuNPs. Consequently, it was found that AuNPs use ClME for the internalization as cell type dependent manner and this cell type dependent ClME can be tracked by SERS. With the use of specific spectral bands changing with ClME inhibitor treatment, ClME of AuNPs could be interpreted by SERS.

As the second pathway, caveolae mediated endocytosis (CaME) was inhibited by Nys and Fil. Even both of the inhibitors did not cause severe toxicity on the cells like Scr; Nys caused dose dependent slight toxicity on healthy cell lines PNT1A and Beas-2b cells. When uptake inhibition ability of two inhibitors was compared, controversial results were obtained. Nys caused uptake inhibition in all cell lines with different decrease rates while Fil causes smaller uptake inhibition in cell lines except for HeLa cells. When obtained spectral changes with Nys and Fil treatment were investigated, significant changes were observed with the treatment due to the elimination of CaME for the internalization of AuNPs. With the removal of cholesterol from the lipid raft, CaME could not be started from the lipid raft domains of the membrane that when compared with the control groups, events occurring during the CaME such as membrane invagination, pit formation, vesicle formation, caveosome formation cannot occur thus, obtained spectra significantly affected as the altered intensity changes in the protein, lipid corresponding peaks. Moreover, spectral changes were found as cell type dependent similar with the other inhibitors which could be the result of different nature of cells, differential uptake rate of AuNPs and different protein corona structure on the AuNPs. For Fil treated cells, healthy cell lines PNT1A and Beas-2b was found more affected than cancerous A549 cell line and most affected cell line was PNT1A cell line with least amount of AuNPs internalized. Moreover, when Nys treatment was compared with Fil treatment, Fil was found more effective than Nys. This could be caused by the side effects of Fil including disruption of cell permeability, disruption of the F-actin linkage with plasma membrane. When obtained SERS spectra is compared with the spectra obtained after the elimination of ClME, differences in were obtained which shows that SERS can distinguish different type of endocytosis pathway that AuNPs use in the same cell line.

As the third pathway, macropinocytosis was inhibited by CytoD and Aml. Both of the inhibitors did not cause any cytotoxicity on the cell lines and CytoD caused inhibition of AuNPs uptake only on Beas-2b cell line and Aml inhibited AuNPs uptake at highest dose

for Beas-2b and PNT1A cell lines. When obtained SERS spectra is investigated most dramatic changes were observed due to the hindering actin polymerization. When actin polymerization is blocked, it affects macropinocytosis in different steps. In the first steps, actin filament formation starts membrane invagination and vesicle formation and it requires recruitment of different proteins to the endocytic site. On the other hand, actin is also used for the movement of the formed vesicle, fusion with endosomes and formation of late endosomes and endolysosomes. Thus, with the inhibition of many different steps, dramatic and significant changes were observed on the obtained spectra. Beas-2b cell line is affected most because cancerous A549 cells were more resistant to the side effects of the selected inhibitors than healthy Beas-2b cells. On the other hand, when compared with other inhibitor treatments, most dramatic changes were observed with CytoD treatment due to the inhibition of actin polymerization which is used in different type of endocytosis pathways. Actin is mostly used in macropinocytosis but it still responsible vesicle movement and fusion in other pathways. However, these dramatic changes could be used for the comparison of micropinocytosis with other pathways and use as marker for the interpretation of micropinocytosis.

As the fourth pathway, all receptor mediated endocytosis pathways were inhibited by NaN₃ and dG. NaN₃ caused dose dependent toxicity on all cell lines while dG did not cause any toxicity. NaN₃ caused toxicity lead induction of not only early and late apoptosis but also necrosis on all cell lines. For the inhibition of the AuNPs uptake, both of the inhibitors decreased the uptake in all cell lines with differential decrease rates. ATP amount was also examined due to the use of ATP depletion agents and ATP depletion was observed significantly for Beas-2b cell line and A549 cell line with dG. It should be taken into account that these ATP depletion agents are differ from other inhibitors because by depleting ATP, they inhibit all type of receptor mediated endocytosis and also interfere with many different cellular metabolisms due to the mechanism of action. When obtained cellular SERS spectra were compared, effect of both agents could be considered as related not only interrupted endosomal pathways but also effects in other cellular metabolisms such as cytoskeleton remodeling, mitochondrial activity, glycolysis, and cellular morphology. For instance, intensities of some of the peaks were found to altered in all SERS spectra regardless of cell and treatment type with both NaN₃ and dG treatment which could be caused by the recruitment fail of the proteins to the endosomal region

required for the maturation of early endosomes to late endosomes and endolysosomes. On the other hand, NaN₃ and dG specific spectral changes were also observed with the treatment. These results showed that even with the same inhibition purpose as ATP depletion, metabolic effects of two different agents could be discriminated by SERS and effects not only on the endosomal pathway but also other cellular metabolisms could be tracked by SERS.

As the third examination, origin of the intracellular SERS spectra was investigated by isolating AuNPs filled endosomes and comparing SERS spectra with whole cell SERS spectra. Isolation of endosomes was achieved by sucrose-Percoll gradient ultracentrifugation and during isolation, nuclear and endosomal fractions were also obtained and their SERS measurements were achieved. When time dependent spectral changes in the endosomes, nuclear and cytosolic fractions were investigated, it was found that SERS signal can be achieved even from the 15 min exposure of cells with AuNPs. With increasing time, obtained spectra changed which is directly related with the localization and microenvironment of the AuNPs. With changed surrounding environment, spectral changes were observed which shows SERS spectra significantly interpret the time dependent route of AuNPs. When cells treated with AuNP more than 4 h, AuNPs are started to be accumulated in the late endosomes and also starts to escape from endosomes and spread through both cytoplasm and nucleus and SERS signal intensity increased not only in the endosomes but also in the cytoplasmic and nuclear fractions. This information could be used for the interpretation as the presence of AuNPs in the nuclear and cytoplasmic fractions. Moreover, when colors of the fractions were compared, purple color of the fractions also provided the presence of AuNPs in the fractions. This information could be used to deduce that AuNPs could be found in the cytoplasmic and nuclear fractions. Furthermore, when obtained fractional SERS spectra are compared with the whole cell spectra, resemblance to the all fractions were observed and some of the peaks were found in both fractions and whole cell spectra. All of these results could be used for the deduction as that intracellular SERS spectra is composed not only from the AuNPs reside inside the endosomes but also the AuNPs escaped from the endosomes and found in cytoplasm and nucleus.

Lastly, to observe the capability of the SERS to interpret intracellular metabolism rather than endocytosis, macrophage activation was investigated by SERS. For this purpose, LPS

was used for the activation of macrophages through M1 type and activation process was examined by nitrite production. After macrophages were activated, AuNPs response of activated macrophage was also investigated. To examine the activation mechanism of macrophages, LPS and AuNPs given to the cells at the same time and SERS spectra was achieved under increasing concentrations of LPS and it was noticed that the peak intensities originating from proteins, phospholipid and phosphatidylinositol were found to be changed significantly which is caused by the M1 type activation mechanisms. After activating macrophages, they were also exposed to increasing concentrations of AuNPs to enhance phagocytosis, and when obtained spectra was compared to simultaneous treatment of LPS and AuNPs, more dramatic changes were obtained in most of the peaks originating from not only proteins but also membrane structures including cholesterol and phospholipid and also nucleic acids which could be related with the enhancement in the phagocytosis process.

Consequently, it could be said from the present work that SERS could be used to assess NMs endocytosis route from single living cells without extensive sample preparation or labeling. However, when intracellular SERS spectra is interpreted, protein corona structure on the NPs should be considered, because PrC structure on the NPs changes by the cell culture conditions and these changes alter uptake amount and route of AuNPs which are directly reflected in the obtained intracellular SERS spectra. Moreover, SERS could discriminate endocytosis pathway dependent changes in cell dependent manner and not only endocytosis dependent changes but also side effects of the used inhibitors could be also tracked by cellular SERS spectra. Furthermore, when origin of the cellular SERS spectra was examined, it was found that obtained intracellular SERS spectra is not coming only from endosomes. It was found that AuNPs escape from the endosomes and found in both cytosol and nucleus where interact with the cytosolic and nuclear biomolecules and when these molecules come close vicinity to the AuNPs, these interactions are reflected in the SERS signal. Escape of the AuNPs could be even tracked at 15 min treatment with distinct SERS peaks at cytosolic and nuclear fractions. On the other hand, for the comparison of the whole cell spectra, it was revealed that some of the peaks found in the whole cell spectra can be also found in different fractions. This data shows that obtained intracellular SERS data is not obtained only from the AuNPs inside the endosomes but also from the escaped AuNPs which can be found all parts of the cell including cytosol and

nucleus when 50 nm AuNPs were used. Lastly, when use of SERS for the investigation of macrophage activation mechanisms examined, it was found that SERS could discriminate LPS triggered activation of macrophages in dose dependent manner and SERS could also be used to interpretation of activated macrophage response to the AuNPs.

Evaluation of the endocytosis of AuNPs could be continued with further investigations based on different parameters. While in this study only one type of AuNPs with approximately 50 nm size was used, with changing NMs based properties such as shape, surface charge and chemistry, endocytosis mechanisms, protein corona conformation and thus all fate of NMs could be altered and examined by SERS. To understand deeply the SERS signal obtained from endosomes, different type of endosomes including early endosomes, late endosomes and endolysosomes could be separated and differences coming from them examined by SERS regarding to the different properties of these endosomes. The investigation of the immune response to the AuNPs, evaluation of activation mechanism could be expanded by other type of macrophage activation processes such as M2a or M2b and also other immune cells and their activation mechanisms could be examined to observe immunological mechanisms by SERS.

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