

BIOMATERIAL-INTEGRATED METASURFACES FOR BIOMARKER DETECTION

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By
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Biomaterial-Integrated Metasurfaces for Biomarker Detection

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We certify that we have read this thesis and that in our opinion it is fully adequate,
in scope and in quality, as a thesis for the degree of Master of Science.



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ABSTRACT

BIOMATERIAL-INTEGRATED METASURFACES FOR BIOMARKER DETECTION

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Today, healthcare system is in the midst of a crucial transition from centralized care to self-monitoring approaches, aiming to reach more individuals; to reduce the workload in hospitals; and to minimize healthcare costs. In this regard, biosensor platforms play a mainstay role in disease diagnostics as an alternative or complementary to current interventions. However, integrating existing biosensing systems into the point-of-care (POC) settings is mostly hampered by the need for bulky instrumentation, lengthy assay procedures, and high-cost platforms. In this thesis, we aim to develop a new biosensing strategy that integrates biomaterials with metasurfaces for this manner. To achieve this aim, optical disks were recycled to obtain their inherent nanograting surfaces as the plastic substrates. Accordingly, two different types of metasurfaces were fabricated (i) by coating the plastic substrates with titanium (Ti), silver (Ag) and gold (Au), and (ii) the latter one was coated with Ag only. Poly-L-Lysine (PLL) was then integrated onto these surfaces to construct a biopolymeric adlayer. To enhance sensing characteristics, biomaterial (PLL)-integrated metasurfaces were decorated with gold nanoparticles (AuNPs), and also, nanoislands (NI) were formed on these surfaces through a chemical reduction reaction of chloroauric acid and hydroxylamine hydrochloride. To benchmark analytical performance of sensors, bulk sensitivity analysis was performed with 1%-70% glycerol solutions. In addition, exosomes were employed as model biomarkers in this study since they are released from kidneys to renal space dynamically, and they carry critical information on disease conditions, holding pivotal impact to be employed for early diagnosis of kidney diseases. With the aforementioned sensing scheme, minute concentrations of exosomes were detected over a wide-sensing area decorated with either anti-CD81 antibodies or anti-CD63 antibodies. In sum, we anticipate that this platform would resolve overwhelming challenges in cost- and complex fabrication-related

challenges, and consequently, it would offer an affordable and facile-to-use diagnostic platform for this realm.



Keywords: Optical Disks, Nanoplasmonic Biosensor, Enhancement, Nanoisland, Exosome Capture.

ÖZET

BİYOBELİRTEÇ TESPİTİ İÇİN BİYOMATERİYAL ENTEĞRE EDİLMİŞ META YÜZEYLER

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Malzeme Bilimi ve Nanoteknoloji, Yüksek Lisans

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Günümüzde sağlık sistemi, daha fazla bireye ulaşmayı, hastanelerdeki iş yükünü en aza indirmeyi ve sağlık harcamalarını azaltmayı hedefleyen; merkezi bakımdan kendi kendini izleme stratejilerine önemli bir geçişin ortasındadır. Bu bağlamda, biyosensör platformları, mevcut müdahalelere alternatif veya tamamlayıcı olarak hastalık teşhisinde temel bir rol oynamaktadır. Fakat, mevcut biyosensör sistemlerinin hasta-başı uygulamalara entegre edilmesi, çoğunlukla devasa cihazlar, uzun test prosedürleri ve yüksek maliyetli platformlar ihtiyacı nedeniyle engellenmektedir. Bu tezde, biyomalzemeleri metayüzeylerle bütünleştiren yeni bir biyosensör stratejisi geliştirmeyi hedefliyoruz. Bu amaca ulaşmak için, optik diskler, plastik kalıp olarak kullanılarak kendilerinde bulunan nano-ılgara yüzeylerini elde etmek için geri dönüştürüldü. Buna göre, (i) plastik kalıpların titanyum (Ti), gümüş (Ag) ve altın (Au) ile kaplanmasıyla ve (ii) sadece bu kalıpların Ag ile kaplanmasıyla iki farklı yüzey oluşturuldu. Biyopolimerik katman oluşturmak için daha sonra yüzeye Poli-L-Lizin (PLL) entegre edildi. Algılama özelliklerini geliştirmek için biyomateriyal (PLL)-entegre edilen metayüzeyler altın nanoparçacıklar (AuNP) dekore edildi ve ayrıca bu yüzeylerde kloroaurik asit ve hidroksilamin hidroklorürün kimyasal indirgeme reaksiyonu ile nanoadalar (NI) oluşturuldu. Sensörlerin analitik performansı değerlendirmek için %1-%70 gliserol çözeltileri ile çevre duyarlılığı analiz edildi. Ek olarak, eksozomlar, böbreklerden böbrek boşluğuna dinamik olarak salındıkları; hastalık koşulları hakkında kritik bilgiler taşıdıkları ve böbrek hastalıklarının erken teşhisi için kullanılabilecek çok önemli bir etkiye sahip oldukları için bu çalışmada model biyobelirteçler olarak kullanıldı. Yukarıda bahsedilen teşhis dizaynı ile anti-CD81 antikorları veya anti-CD63 antikorları ile dekore edilmiş geniş algılama alanı üzerinde çok küçük eksozom konsantrasyonları tespit

edildi. Özetle, bu platformun maliyet ve karmaşık fabrikasyonla ilgili karşı konulmaz zorlukları çözeceğini ve akabinde bu alanda kullanılabilecek uygun fiyatlı ve kullanımı-kolay bir teşhis platformu sunacağını umuyoruz.



Anahtar sözcükler: Nanoplazmonik Biyosensör, Metamateryal, Nanoada, Eksozom, Böbrek Hastalıkları .

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Chapter 1

Introduction

1.1 Significance of Point-of-Care Testing

Human being has been dealing with so many diseases, both mild and severe types, and the role of diagnostics is crucial along with appropriate and timely care for individuals and public health [9]. Mostly, conventional diagnostics such as molecular- and microbiology-based assays require high-cost and sophisticated instruments, and they are limited to full-fledged facilities for proper testing. Although these techniques offer accurate and precise results, they do not provide adequate and rapid diagnosis, particularly in an emergency, ambulatory, and remote settings [10]. To address these limitations, comprehensive studies have been conducted to create alternative strategies. Advances in technologies provide various solutions for early diagnosis—in particular, point-of-care testing (POCT) is the most common approach since it enables a viable shift from traditional diagnostic tests in clinical laboratory settings to bed-side or home-settings [10, 11]. The primary benefit of POCTs is the determination and quantification of the target analytes near to individuals, including better disease diagnosis, monitoring, and management in timely fashion, which improves and accelerates healthcare opportunities for society [12].

Within time, the scope and definition of the POCTs have evolved [13]. In the early phase, POCTs were described as tests performed at or close to the patient [13]. Considering the need for clinics and society, the POCTs are considered as the tests that can be performed on a demand and the results of these tests provide or guide a clinical decision, thereby taking an action to provide an enhanced health outcome [13]. In sum, the primary requirement of these definitions is that POCTs should be performed with near-patient and short turnaround for the results [13]. In addition to these parameters, World Health Organization (WHO) has been designated standards for the ideal test called ASSURED (affordable, sensitive, specific, user-friendly, rapid, equipment-free, and delivered) while developing POCTs. Over time, scientific community has proposed two additional criteria: (i) real-time monitoring and (ii) ease of sample collection to the current ASSURED criteria, converting it to REASSURED [14]. Real-time connectivity is included to broaden the geography of tests through a mobile reader or a mobile phone, thereby transmitting necessary data to decision-makers simultaneously. Ease of specimen collection is also another new parameter to minimize invasive clinical interventions for obtaining the specimen [14].

From an economical perspective, POCTs do not only offer benefits in terms of diagnostics, but also take part in a noteworthy market share in medical devices. The expected growth rate of the global POCT market is from US\$ 23.16 in 2016 to US\$ 36.96 billion in 2021 at a compound annual growth rate (CAGR) of 9.8% from 2016 to 2021 [12]. North America dominates to POCT market, and Europe follows. The expected CAGR of the Asia-Pacific countries is 14.2%. Regarding the market share growth, the development of POCT is currently a hot topic for not only clinics and population health, and also, for economics. To further improve POCT, many platforms and strategies (e.g., microfluidics, device automation, and signal readout systems) are integrated [12]. Among them, biosensors are the integrative modality in developing POCTs since they enable the improved limit of detection (LOD) [15] and sensitivity [15]; they can be miniaturized into portable setups [16]; they can be adapted easily to many diseases or targets [6]; they can be employed with a wide range of sample source (blood, serum, urine, saliva, sweat, etc. [15]) to detect several kinds of target molecules

(proteins, nucleic acids) [17].

1.2 Biosensor

Biosensing platforms are the basis of POC applications, particularly in disease diagnosis. They enable and offer rapidity, cost efficiency, easy-to-use, high sensitivity, and selectivity [15]. The classical approach described it as “*analytical devices that convert biomolecular interactions to readable signals by utilizing five different components in general* [15].” These components are analytes/targets (protein, metabolites, nucleic acids, human cells, microbes/pathogens, drugs, and other small molecules [18]), bioreceptors (antibody [19], enzyme [20, 21], peptide [22], cell [23], aptamers [24], molecularly imprinted polymers (MIPs)[25, 26]), transducers (e.g. electrochemical [27], optical [28, 29, 30], mechanical [31], lateral flow assays (LFA)[32]), electronics and display [15]. Considering these components, biosensors are generally categorized based on biorecognition elements and transducer types [33]. These transducers can be in the form of thin film or nanoparticles made up of metals (gold [34, 28, 35], silver [36, 37, 38], titanium dioxide [19] and platinum [39, 40]), a semiconducting material (silicon [41] and quartz [41]) or nanomaterials (carbon [42]).

To assess the performance of biosensing platforms, sensitivity, the LOD value, linear dynamic range, reproducibility, precision of the response, selectivity, rapidity, operational and storage stability, simple usage, and compactness are considered as main criteria [43]. The enhancement of these terms can be accomplished through by a combination of functional nano [44] and smart materials [45, 46], carbon-derived materials for instance carbon nanotubes or graphene [47], and metallic nanoparticles most commonly gold nanoparticles [48] and silver nanoparticles [49, 36].

Optical biosensors are one of the most commonly employed principles in biosensing and POCTs. They display remarkable performance in detecting and

monitoring biological analytes and biomolecular interactions, as well as in providing considerable advances in clinical diagnostics, drug discovery, and food industry [50, 51]. Moreover, optical-based biosensors offer a variety of benefits for instance; high sensitivity and selectivity, robustness, reliability, real-time monitoring, and label-free detection [50, 51, 52]. They can be developed and utilized with different principles; for instance, plasmonics (e.g., surface plasmon resonance (SPR) and localized surface plasmon resonance (LSPR), Surface-enhanced Raman scattering (SERS), surface-enhanced fluorescence and surface-enhanced infrared absorption spectroscopy) [161]. Plasmonic-stemmed systems have gained high interest in biosensor development because these platforms empower the quick response of bio-analytes in specimens and offer high sensitivity and selectivity in contrast to currently available instruments simultaneously [49].

1.3 Plasmonic Based Biosensors

Basically, plasmonic biosensors quantify or monitor the binding of molecules, therefore providing information on binding affinity and kinetics [53]. This strategy mostly track the changes in the index of refraction (RI) in real-time [54, 55]. Plasmon resonance nanostructures have attracted a significant interest in the scientific community because of their excellent optical properties. Resonance is a critical parameter that determines sensitivity and specificity, yet there must be precise operations in designing, manufacturing, and optimizing plasmonic substrates [56]. They usually employ metals as plasmonic substrate; on the other hand, new integrations (e.g. such as graphene and its derivatives) to plasmonic materials are also attracting attention in the field [57]. SPR, LSPR, and SERS are widely employed plasmonic modalities. The power of SPR and LSPR in clinics (e.g., diagnostics), environmental monitoring, and food safety as a biosensor platform lies in the fast and sensitive detection of various analytes (viruses, bacteria, proteins, etc.) [6, 53]. Similar to the, SPR and LSPR, SERS based plasmonic platforms promises label-free, sensitive, detection by using an enhanced local electric field on the plasmonic material surface when monochromatic light is employed for excitation [6].

Plasmonic biosensors can be investigated under the scope of chip-based plasmonic sensors and colloidal nanoparticle-based plasmonic sensors [58]. These two categories can also be considered platforms that utilize thin metallic film and inorganic plasmon resonant nanostructures [59]. The principle of thin metallic film-based systems is referred to as SPR-based biosensors, and plasmon resonant structures utilize LSPR-based biosensors [59]. In addition, plasmon-enhanced fluorescence (PEF) sensors, a.k.a. surface plasmon-enhanced fluorescence spectroscopy (SPFS), surface-enhanced fluorescence spectroscopy (SEFS), or metal-enhanced fluorescence (MEF), have been introduced to the field by utilizing chip-based plasmonic sensors [59].

1.3.1 Surface Plasmon Resonance (SPR)

Surface plasmon resonance (SPR) is an optical phenomenon relying on the coherent oscillation of electrons between plasmonic metal and dielectric media (at their interface) by exciting with electromagnetic radiation [49, 60, 61]. SPR utilizes surface plasmon polariton (SPP) waves to monitor the interactions between analyte and sensor surface. SPP refers to the perpendicular confinement of evanescent electromagnetic waves propagating at the interface between a metal and a dielectric surface [49]. In this regard, the principle of the SPR can be explained as propagating light to an electrically conductive gold layer at the interface between two media which have different refractive index under total internal reflection (TIR) conditions, e.g., a glass of a sensor surface (high refractive index) and a buffer (low refractive index) [62]. The RI near the metal surface is changed locally proportional to biomolecule concentrations. As a consequence of the RI changes, the propagation constant of SPP is also be altered, and this can be optically measured via the attenuated total reflection (ATR) method [62]. The measurement is taken place at the sensor surface (200–300 nm) in the form of RI change. Rate of interactions (e.g., association, dissociation, or both) to obtain kinetic rate constants, analyte concentration, and binding level to obtain affinity constants are different information that can be attained by SPR [63].

The excitation of the surface plasmons matches the momentum of incident light (an electromagnetic wave) and plasmon modes to achieve charge-coupled oscillations. Different configurations can be employed for excitation; for instance, prism, grating, waveguide, photonic crystal, and fiber-optic-based coupling [49]. The resonance peak wavelength is tuned by the shape or structure of materials used, such as solid or hollow structures [60]. SPR-based biosensor enables robust, label-free, real-time, and susceptible detection in diverse fields including environmental monitoring, biotechnology, clinical diagnostics, drug discovery, food safety, and security-based on the binding-induced refractive index change [64, 65, 66, 63, 62].

1.3.2 Localized surface plasmon resonance (LSPR)

Localized surface plasmon resonance (LSPR) is another optical phenomenon, which is similar to the principle of SPR. Still, LSP modality is restricted to smaller volumes, such as nanoparticles or nanostructures with smaller sizes than the incident wavelength [61, 67]. The nanoparticle size, shape, and material are the determinants for the wavelength (e.g., visible or near-infrared) of the LSPR [68]. In this case, the free electrons of particles oscillate collectively and create localized surface plasmons, which have enhanced electric fields at the surface and decay with respect to distance. In addition, noble nanoparticles indicate maximum optical extinction at the plasmon resonant frequency while visible wavelength is applied. The surrounding medium's RI influences the optical extinction utilized for the sensing strategies [69]. The resonance generates a spectral peak that can be obtained from absorption or scattering and strong electromagnetic near-field enhancements [69]. These resonances are related to noble metal nanostructures since the addition of nanoparticles, nanostructures (near to metal surface) enable local plasmon oscillations [69, 61]. On the other hand, the LSPR-based biosensor platforms offer various advantages, including eliminating coupling modules like prisms. In addition, in the measurement systems, angle of the incident light is not as critical as in SPR-based platforms, and therefore, they can be fabricated in more affordable and flexible designs [61].

1.4 Metamaterials

Metamaterials are artificially fabricated materials that are highly utilized in fabricating plasmonic sensors because of their light controlling capability and intrinsic losses [34, 35]. They are fabricated by arranging materials with plasmonic properties in the wavelength thickness or nanostructures that are spatially arranged meta-atoms [34]. These nanostructures could be either periodic or special arrangements of metal/dielectric elements in subwavelength dimensions [70]. They are artificially created by periodically arranged structures mostly made of noble metals or high index dielectrics [71, 72]. The arranged constituents materials do not exploit their properties; metamaterials provides different properties from their substances [70]. They denote optical magnetism, negative reflections, electromagnetic induced transparency, bianisotropy, and Fano resonance [34]. Therefore, light properties (e.g., phase, amplitude, and polarization) can be controlled due to the structure of a periodic structure, array of optical scatterers, and subwavelength distance between them [34, 70].

The optical metamaterials are mainly made up of noble and plasmonic metals, semimetals, and intermetallic compounds (e.g., metal nitrides, hydrides, oxides, borides), and transparent conducting oxides, and dielectrics [70]. The common metal material for plasmonic metamaterials is gold, silver, aluminum, and copper since they provide resonance in the visible and mid-IR range. They exploit crucial advantages such as well-known fabrication processes; silver and aluminum indicate plasmonic properties in the UV range; gold is a biocompatible material [70]. In addition to these common metals, titanium nitride (TiN) and zirconium nitride (ZrN) are also employed for plasmonic and their frequency is in the visible range of light [70]. Moreover, graphene exploits huge potential due to its excellent electrical features that are coupled with optical features along with a surface plasmon support at lower terahertz frequencies and mid-infrared, enabling high confinement [73]. Integration of graphene with metasurfaces increases the interactions between incident light and graphene, thereby enhancing sensitivity [73].

1.5 Nanomaterials

Nanomaterials are also termed nanostructured materials within the range of nanoscale (~ 100 nm or less). The main property of the nanostructured materials is controllability and manipulation of the structure to attain desired behavior. Particles with at least one dimension at a nanoscale are also considered nanostructured materials that exploit unique and tunable properties; for instance, high surface-volume ratio, easy derivatization, and exclusive thermal, mechanical or electronic properties [44]. One of the benefits of employing nanomaterials is achieving a high surface area in comparison to macroscale materials with low volumes [74]. In addition to nanomaterials, smart materials have also been integrated into the sensor platforms due to their tunable properties such as thermo, light, magnetic or pH responsiveness [45, 46].

1.5.1 Nanostructures and Nanograting

The recent progresses in the semiconductor industry mediates innovative fabrication techniques to produce micro or nanostructures in electronics, optics, mechanics, biology and chemistry [75]. Especially, metallic nanostructures are vital integral sections of the plasmonic sensors [76]. The prevalence of the nanostructures in the field has evolved due to the various fabrication techniques, which are basically categorized into two approaches: (i) top-down (e.g., lithography techniques such as photolithography, nanoimprint or electron-beam lithography, thin film deposition such as chemical vapor deposition or epitaxial growth, etching and metallization) or (ii) bottom-up (e.g., self-assembled monolayers, colloidal lithography, and layer-by-layer assembly) [75, 76]. Although substantial progress is acquired, fabricating the nanoperiodic structures maintains its complexity by the deficiency of precise control on manipulating light in the visible spectrum [77]. In addition, electron beam lithography (EBL) and focused ion beam (FIB) milling are costly processes, and they do not allow large patterning areas with high production [76]. On the other hand, nanoslits, nanoholes, gratings or nanoparticles are well-employed nanostructures in plasmonic sensors [76].

Besides their drawbacks (e.g., complex fabrication), grating structure enables to minimize the sensing area [76]. New strategies such as the modification of already existing nanostructures to tune the resonance wavelength in plasmonic sensing [35], notably minimize the need for complicated and expensive fabrication of the nanograting structures.

1.5.2 Nanoparticles

Nanoparticles, especially gold nanoparticles (AuNPs), are widely studied and employed types of nanomaterials in the medical area and biosensing realm [78]. They have substantial properties, including ease-of-functionalization and excellent optical properties, which can be modified by changing their size, shape, composition, and arrangement or coupling with other particles that have plasmonic properties [79]. They can be used alone as a signal provider and/or combined with different plasmonic structures to enhance optical signals [80, 28, 81]. As explained in the previous sections, different plasmonic sensing strategies (e.g., SERS, LSPR, calorimetric, etc.) and AuNPs can be integrated into all these strategies [79]. Controlling inter-particle distance, their concentrations, and morphological properties (e.g., rod, cube, cage or star) enables to control the plasmonic signal [82, 83].

In addition to gold (Au), a variety of metals (aluminum (Al), copper (Cu), lithium (Li) and silver (Ag)) creates surface plasmons in visible (vis) and near-infrared regions (NIR) [84]. Among them, Au is one of the mostly-applied plasmonic materials. On the other hand, Ag is an important alternative to Au due to its ability to exhibit sharper angular resonance, which enhances the sensitivity [85]. In the plasmonic sensors, Ag provides sharper and higher plasmon resonance signals, as well as it can absorb and scatter the light ranging from 300 to 1200 nm [61]. Despite all these advantages in the use of Ag for plasmonic sensing, chemical stability is much lower compared to the Au-stemmed surfaces, which hinders the utility of Ag in biosensing applications and long-term storage [85]. There are three different strategies for the preparation of Ag nanostructures:

(i) chemical synthesis (Citrate Reduction, Silver Mirror Reaction, Polyol Process, Seed-Mediated Growth, Light-Mediated Synthesis), (ii) template-directed growth, and (iii) lithographic fabrication [84].

1.5.3 Impact of the Nanoislands on Plasmonic Sensing

In plasmonic sensors, the enhancement of sensitivity is achieved with several kinds of approaches, including labeling, integration of nanomaterials (e.g., graphene, nanoparticles, etc.) or nanostructures (e.g., nanogratings, nanoholes, nanopores, nanowells, nanotubes or nanoislands (NI), etc.) [86]. The nanostructures, particularly NI, come forward since they create nano-gap-based plasmonic hot spots to boost the sensitivity fabrication [87]. There are different fabrication techniques of NI that include conventional nanolithographic methods and dewetting. The limitations of these methods are time-consuming, high-cost, low fabrication yield or prone to interpersonal errors in the fabrication [87].

In this regard, chemical synthesis is an alternative method for NI production [87]. Briefly, chemical growth relies on the solution-based synthesis of gold nanoparticles; therefore, nanoparticles or nanoislands can be grown on the substrate or nanostructure. Similar to nanoparticle synthesis, the common pathway in NI synthesis utilizes the seeding of particles. These NI structures are mainly used for the PEF or SERS strategies [88]. Chemical synthesis of NI on the substrate can mainly be performed with three steps: (i) adding ammonium hydroxide into a chloroauric acid, (ii) the addition of sodium borohydride, and lastly, (iii) hydroxylamine reduction in a chloroauric acid solution [88, 89]. The structure of the NI and their optical properties can be further tuned by the changing seed density, shape of particles or their concentration in the solution [88].

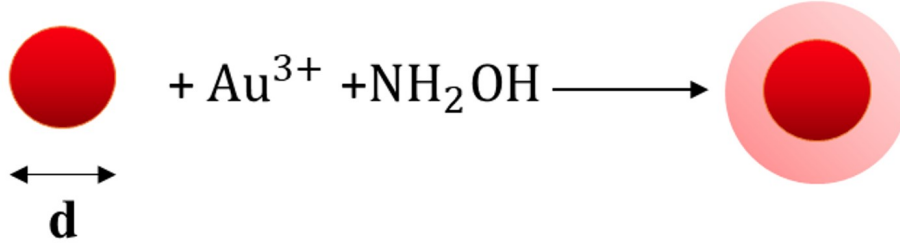


Figure 1.1: A chemical reaction for seeding of AuNPs [1].

1.6 Functionalization and Biorecognition Element

As aforementioned, biorecognition element is one of major sections in biosensors. Besides the function of biorecognition elements, their immobilization onto a transducer also impacts the analytical performance. To immobilize a biorecognition element onto the sensor, surface chemistry surface chemistry and bioconjugation techniques are widely applied to achieve their uniform and proper orientation [90, 3, 91]. Different classified approaches have been performed, such as reversible and irreversible, chemical or physical methods.

Physical methods are composed of adsorption and encapsulation (entrapment), and chemical methods contain crosslinking and covalent bonding methods, which are more preferable due to firm and stable binding of the bioreceptor onto the surface [90, 92, 93, 85]. Besides bioreceptors, the integration of smart materials (e.g., nanomaterials, nanoparticles) to a transducer via surface chemistry is also commonly applied to modulate analytical performance. Choosing the proper surface chemistry requires the molecular-level understanding and chemical properties of the surface [94]. In sum, surface chemistry is one of the crucial steps while designing and developing a biosensing platform [92].

For the plasmonics, self-assembly coupling, covalent coupling using chemical

linkers, and bioaffinity interaction coupling are the main categorization of surface functionalization [3]. Oriented self-assembly coupling is referred to as the formation of disulfide bridges between gold or silver surface. Linker-mediated coupling is executed through 2 or more steps since it requires a chemically activated metal surface [3]. Surface functionalization should be performed first to activate gold surface, then coupling. The common method for Au functionalization is to create self-assembled monolayers (SAMs), providing a suitable reactive end, in particular thiolated organic compounds that form Au-sulfur (Au-S) bonds. In addition to the high affinity in strong Au-S bonds, SAMs decrease non-specific protein adhesion with stable and oriented immobilization [3]. Metal-thiol dative bonds, a.k.a. coordinate covalent bonds, are between thiol-containing molecules and metal ions on metal surfaces. In contrast to regular covalent linkages (sharing an electron by two atoms), dative bonds are formed through two electrons from each atom. Sulfur atoms have unshared pairs of electrons capable of creating a dative bond with a metal atom. In addition to sulfur, disulfides can form a dative bond without requiring prior reduction to thiols. Dithiobis(succinimidyl propionate) (DSP) containing a dithiol bridge, is one of the commonly employed chemical compounds for the activation of metal surfaces or metal particles via dative bonding [2]. In addition to DSP, there are various chemical compounds such as 11-Mercaptoundecanoic acid (MUA), 12-Mercaptododecanoic acid, 1,6-Hexanedithiol, 6-Mercaptohexanol, and 4-Aminothiophenol [95].

On the other hand, after decorating SAMs on the sensors, the presenting groups are critical for the immobilization of the bioreceptors. Mostly amine (amide bond with N-hydroxysuccinimide esters (NHS)), aldehyde (creates imines) or thiol coupling (thioethers) are employed for these purposes [3, 85]. Bioaffinity interactions also offer easy immobilization. Avidin-biotin complex is the most prevalent approach as a bioaffinity interactions coupling. Here, avidin acts as a linker, whereas the bioreceptor (generally antibody) is functionalized with biotin in order to create the interactions [3].

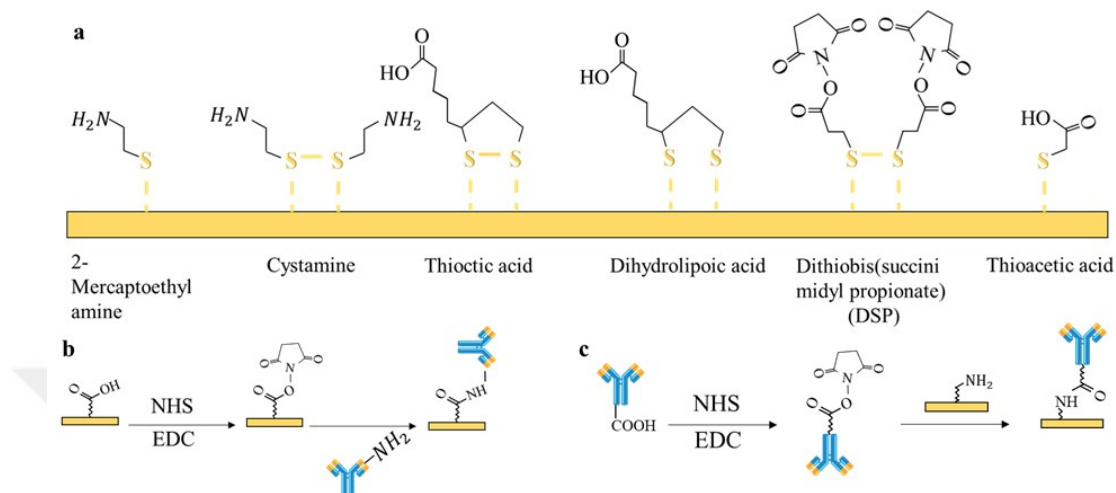


Figure 1.2: Surface chemistry strategies for gold surfaces. (a) Thiol-containing molecules can be used to functionalize gold surfaces. (b-f) Schematic represents the coupling strategies onto SAM-modified gold surface. (b) The lysine residue of antibodies forms an amide bond via N-hydroxysuccinimide esters (NHS). (c) Carboxylic acid group of an antibody (aspartate or glutamate residues) forms NHS ester by reacting with 3-(3-dimethylaminopropyl)carbodiimide (EDC) in the presence of NHS [2, 3].

Chapter 2

Kidney Diseases

Kidney diseases are defined as disorders occurring in kidney structure and function [96]. The severity of kidney disease ranges from mild abnormalities to mortality [96]. If the disease continues for more than three months, it is termed a chronic disease; if it is less than three months, it is referred to as an acute disease [96]. Acute kidney injury (AKI) is a subclass of kidney disease that abruptly reduces renal function within one week [6]. The relationship between CKD and AKI is complicated. If the AKI is diagnosed in the early stage, it is reversible. However, if it cannot be diagnosed early and not treated properly, it progresses to CKD [6]. Chronic kidney disease (CKD) is a clinical syndrome with high morbidity and mortality, and its incidences approximately 10%–13% of the population [97]. It can be defined as kidney damage and GFR reduction ($\text{GFR} < 60 \text{ mL/min/1.73 m}^2$), and its symptoms continue longer than 3 months irreversibly [97, 98]. The damage affects kidney structure (e.g., tumors, malformations, and atrophy) and functions [99]. The kidney dysfunction can be indicated as hypertension, edema, elevated creatinine and BUN levels in the urine [99]. On the other hand, the leading causes of CKD are diabetes and hypertension [100].

Currently, diagnosis of kidney diseases is performed by following guidelines of AKIN, RIFLE or KDIGO by considering serum creatinine (SrC), blood urea nitrogen (BUN), and urine output (UO) levels [4, 101, 102, 103, 104]. Creatinine

is a molecule released from the liver, pancreas, and kidney by synthesizing from amino acids, generally glycine and arginine. It is easily filtered throughout the glomerulus due to its low molecular weight [105]. BUN is also independent from kidney function and easily influenceable by non-renal factors, including protein uptake, catabolic state, upper gastrointestinal bleeding, volume status, or high-dose steroid treatment [102]. On the other hand, the reduction in UO is employed to diagnose AKI, particularly among bedside patients [106]. Although they are considered gold standard parameters in current practice, SrC, BUN, and UO are not adequately accurate since they can be influenced by other organ systems or organs [107]. The inefficiency of commonly used parameters has led researchers to look for alternative molecules released from injured kidneys that state the reduced function in kidneys [108]. These molecules can be constitutive proteins or elevated molecules (e.g., exosomes, miRNA, proteins, and siRNA) as a response to an injury [103].

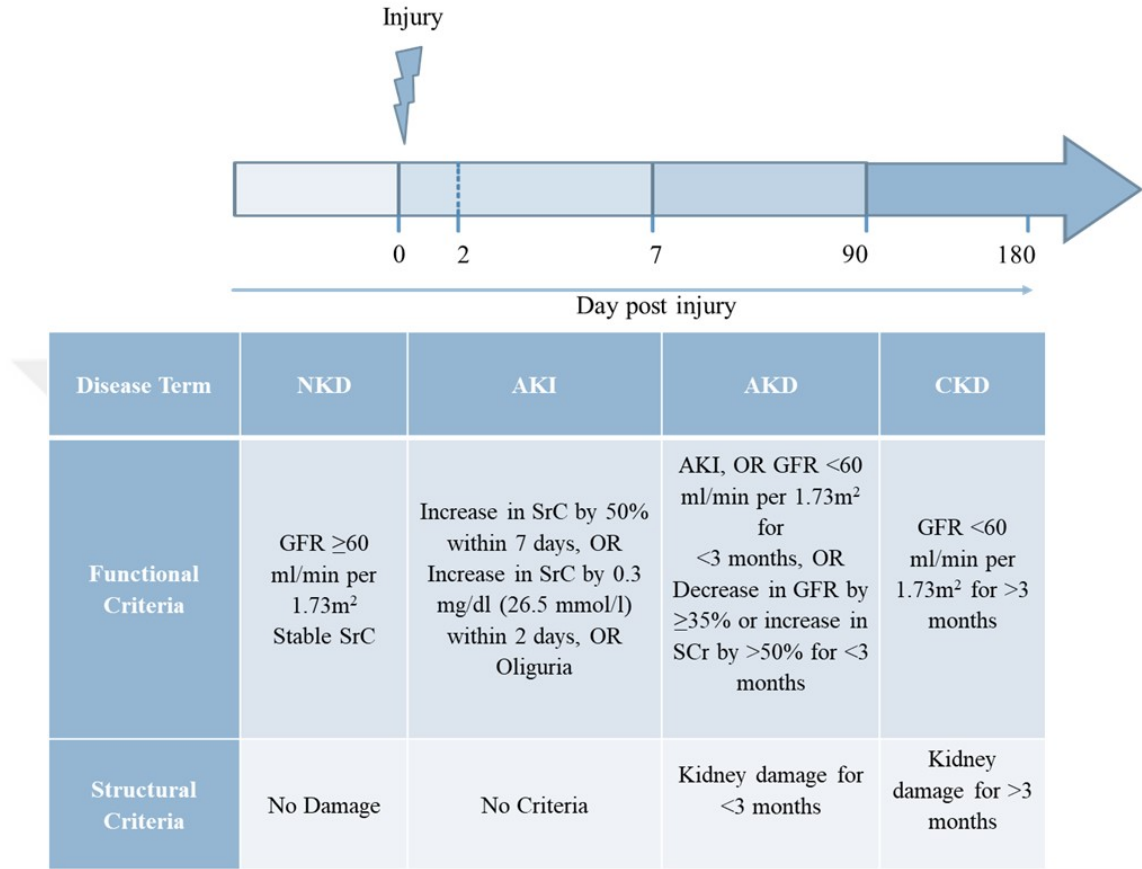


Figure 2.1: The diagnosis criteria of Acute Kidney Injury, Acute Kidney Disease, and Chronic Kidney Disease are presented according to the KDIGO guideline [4, 5].

2.1 Acute Kidney Injury and Biomarkers

AKI is a severe and complex clinical syndrome that occurs due to various causes ending with up abrupt decrease in renal function and accumulation of creatinine, urea, and other waste products [109, 107, 110, 111]. Age [112], gender, race, diabetes mellitus [113], major surgery [114], sepsis, and hypovolemia are the main risk factors for AKI development [115]. One in five hospitalized patients, which can vary based on diagnosis definition, suffer from AKI condition [116]. AKI-related mortality and morbidity create huge concern for public health since

they vary from 20% to 60% in the population [116, 117].

The physiological definition of AKI can be introduced as tubular epithelial cell injury, including endothelial damage and inflammatory cell accumulation due to ischemic perfusion, sepsis, and toxicity of drugs, ending up with tubular apoptosis, necrosis, tubular obstruction, and vasoconstriction [118, 119]. AKI results from reduced renal and intrarenal perfusion, tubulointerstitial inflammation, edema, or diminished filtration capability of the glomerulus [120]. The First 2 days of AKI have crucial importance for recovery, but after 7 days, it may turn into acute kidney disease, a defined duration between AKI and CKD (7-90 days) [4].

Moreover, there are some reports on AKI development during ICU stays of patients who are infected with SARS-CoV-2. AKI involvement is inevitable in hospitalized patients since the human angiotensin-converting enzyme II molecule, where SARS-CoV-2 interacts, is also highly expressed in kidney cells [121, 122, 123]. As reported, 20%-40% of the individuals affected by COVID-19 and admitted to ICU develop AKI, which has been used to evaluate the severity of the disease (e.g., negative prognostic factor, mostly leads to death) [122, 124, 123]. The current diagnosis of AKI is based on creatinine levels; therefore, the statistics may not reflect actual values due to late indication of GFR. In COVID-19 cases, preventive or therapeutic action can be carried out when AKI is involved at an early stage to stop progression or eliminate morbidity and mortality [122, 123].

In addition to its clinical impact, AKI exploits a substantial economic burden on either medical centers and hospitals or individuals. For example, 1% of the National Health Service's budget is spent in England on the estimated inpatient costs related to AKI [116]. In the United States, hospitalization costs have increased from \$5.4 to \$24.0 billion due to the effect of AKI. Severe AKI (e.g., dialysis requiring) requires much more resource utilization, and the cost of hospitalization increases between \$11,016 to \$42,077 per hospitalization compared to non-AKI patients [116]. AKI development creates extended or unplanned intensive care unit (ICU) stays, close monitoring, and extended hospitalization or

early rehospitalization [125].

To diagnose AKI, common biomarkers (e.g., serum creatinine, BUN, and UO) are utilized to determine glomerular filtration rate (GFR) decrease, which is insensitive and results in the late indication of AKI since most injuries are occurred in the tubular epithelial cells rather than the glomerulus [103, 126, 104]. In contrast, inefficiency of serum creatinine, BUN and UO revealed the necessity of novel and more efficient biomarkers, which are released specifically from the injured kidneys, such as constitutive proteins and elevated biomolecules (e.g., proteins, encapsulated molecules in exosomes, miRNA and siRNA) that indicate a decrease in kidney function [108, 103].

In particular, different approaches were employed to classify urinary biomarkers that can be reached non-invasively [126]. Another strategy has been developed to understand functionality and structure in kidneys; for instance, monitoring tubular enzymes and elevated proteins level [126]. Recently, N-acetyl-B-D-glucosaminidase (NAG), neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1) and interleukin-18 (IL-18) are studied extensively for this regard [127]. Moreover, β 2-microglobulin, lysozyme, α 1-microglobulin, light chains, and cystatin C are the other low molecular weight proteins can be easily filtered and re-absorbed in the glomerulus and proximal tubules, respectively. Therefore, they are considered as AKI biomarkers (Figure 2.2).

Briefly, KIM-1 and NGAL biomarkers are considered first-generation biomarkers, and they are produced in tubular epithelial cells after an injury [126]. KIM-1 is a urinary biomarker that can be easily detected from the urine within 12-24 h after the injury. It can be detected from both serum and urine, but detecting this biomarker from urine is more reliable [126]. The studies on NGAL revealed that systemic and urinary NGAL protein levels are low in healthy individuals [128]. NGAL concentration in plasma can be determined in 12 h after the admission to ICU; therefore, AKI (according to RIFLE criteria) can be diagnosed within 48 h [129]. In addition, the ratio of elevation indicates the severity of AKI [128]. The major drawback of NGAL for AKI detection is unspecificity for kidney cells,

and NGAL is also released from the other sources, such as prostate and epithelia of the respiratory and alimentary tracts, potentially resulting in misleading information on AKI [130].

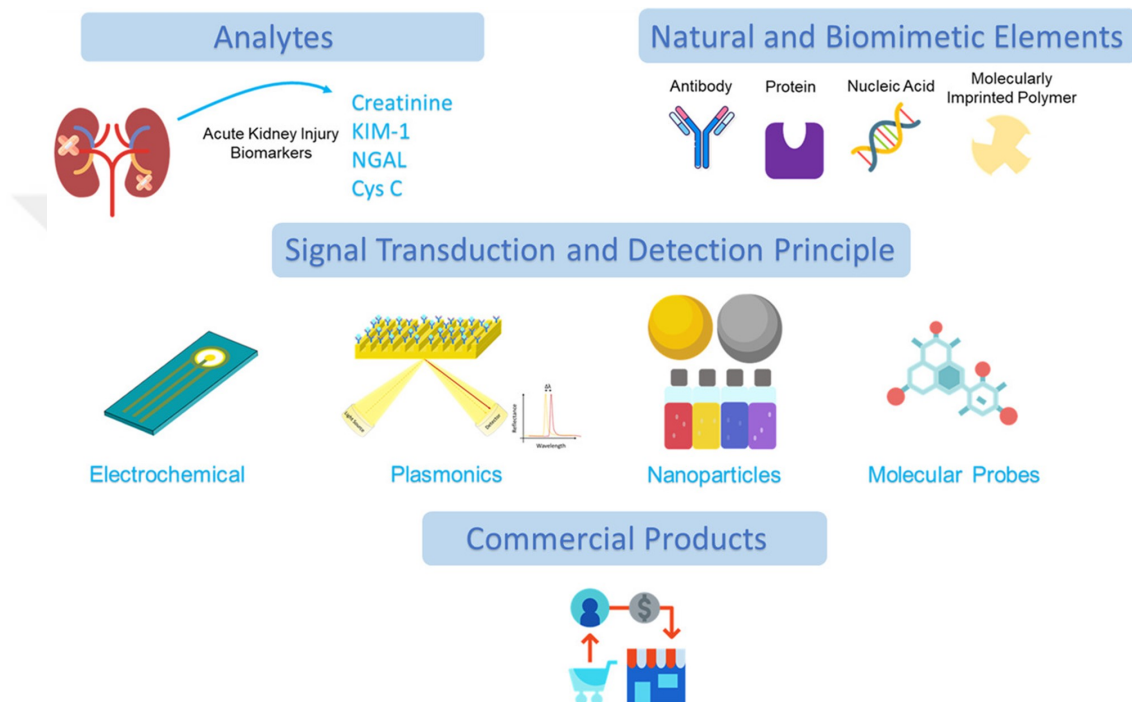


Figure 2.2: A schematic illustrates AKI and its first-generation biomarkers along with some biosensing strategies and examples of commercial products. *Reproduced from [6] with permission from American Chemical Society* (Figure credits: Some icons used on this figure are collected from FlatIcon, and they are modified to the concept accordingly).

Moreover, TIMP-2 and IGFBP7 are urine proteins, which are recently found promising biomarkers for AKI diagnosis [131]. The combination of TIMP-2 and IGFBP7 biomarkers can be defined as second-generation AKI markers [126]. The molecular weight of TIMP-2 is around 24kDa, and the molecular weight of IGFBP7 is 29 kDa [132]. One of the main functions of TIMP-2 is the inhibition of matrix metalloproteinase activity and matrix metalloproteinase-independent effects, and consequently, a significant portion of this inhibition is directly related to the regulation of the cell cycle [133]. They are mostly considered stress

markers since they can be released as a response to non-injurious, noxious stimuli [110]. miRNAs are also included in the second-generation of AKI markers, and they are endogenous single-stranded 19-23 nucleotides obtained from the non-coding region of RNA. miRNAs are responsible for the post-transcriptional level of gene expression regulation, including cell functions, proliferation, differentiation, metabolism, and apoptosis. For a long period of time, miRNA has been related to kidney diseases such as polycystic kidney disease, renal cell carcinoma, diabetic nephropathy, lupus nephritis, and renal allograft rejection [134]. First, miRNA can be a response to injury in a protective behavior or mediator of the developing AKI [135]. The elevation of miRNA in plasma and urine occurs earlier in comparison to conventional biomarkers when renal dysfunction is present [135].

Table 2.1: Recent studies on AKI biomarkers and detection strategies.

Biomarker	Biomarker Releasing Site	Properties	Sample Type	Detection Method	Advantages	Disadvantages	Refs
KIM-1	Proximal tube	States informa- tion on tubular injury	Urine	•ELISA •Lumi- nex- based assay	Elevated urinary levels are highly sensitive and specific for AKI	Mostly con- fused with AKI while the presence of high risk-CKD	[120, 131, 136, 137, 138, 126]
NGAL	Not spe- cific to kidneys only	Reflects tubular damage	•Plasma •Serum •Urine	ELISA Luminex- based assay	The elevation of NGAL re- flects severity Resistant to degradation	Significant- ly affected by age, gender, urinary tract infection, and impaired renal function	[130, 108, 139, 120, 131, 128, 126]

NAG	Proximal tube	Reflects tubular damage and loss of lysosome integrity	•Blood •Urine	Colorimetry	Indicates higher stability compared to other urinary enzymes Investigated under extensive preclinical and clinical status (nephrotoxicity cardiopulmonary bypass, delayed renal allograft function, etc.)	Lysosomal activity or leukocyte degranulation can take place due to an increase in NAG without tubular damage.	[120, 140, 141, 142]
IL-18	Proximal tube	Acute tubular necrosis	Urine	ELISA	Provides the highest efficiency in early stages, especially better interventions for paediatric cases	Potentially affected by endotoxemia, inflammatory and autoimmune diseases	[143, 144, 145]
Cystatin C	Indicates GFR change	Reflects kidney function and GFR	Blood	•Immunoassays •ELISA •Nephelometer	Independent from gender, diet, muscle mass or tubular secretion	Reflects high probability of influencing by glucocorticoids, thyroid function, inflammation, and obesity	[146, 108, 120, 102, 147, 148]

β2-microglobulin	Proximal tubules and glomerulus	Reflects renal tubular injury and function of glomerulus and renal tubules	Urine	•Immunoturbidimetry •ELISA	Helping to stage AKI for determining severity.	Only associated with AKI in paediatric population.	[149, 150]
α1-microglobulin	Proximal Tube	Reflects proximal tubular damage	•Urine •Blood	ELISA	Being stable against various pathological conditions		[151, 152]
Microalbumin	Proximal Tube and glomerulus	Reflects GFR decrease	Urine	Immunoturbidimetry	Predicting the development of AKI without any effect of CKD	Limited to septic cases	[153]
L-FABP	Proximal Tube	Responses to the ischemia and oxidative stress.	Urine	ELISA	•High possibility of being effective endogenous protective antioxidant molecule •Used for AKI diagnosis in children experiencing cardiopulmonary bypass surgery.	•Urinary L-FABP levels are directly not correlated with the severity of AKI •ELISA requires several hours, specialized equipment, and highly trained personnel.	[154, 155, 156, 157]

GGT	Proximal Tube	Reflects injury in brush border	Urine	Colorimetry	low cost and the wide availability of automated assays	Relatively low discrimination of AKI.	[158, 120, 159]
miRNA	No specialized location	Reflects pathology or protection	•Blood •Urine •Plasma	quantitative real time PCR (RT-qPCR)	Do not require complete complementarity stable and abundant in urine and plasma	Affected by sample types	[160, 134, 135]
TIMP-2/IGFBP-7	Distal Tube-Proximal Tube	Induces cell cycle arrest	Urine	Immunoassays	Categorized as a pre-injury biomarker of AKI	Reflects kidney stress rather than the damage	[161, 162]

2.2 Exosomes as a Kidney Disease Marker

Extracellular vesicles (EV) have been released from all kinds of cells, including prokaryotes and eukaryotes, on the course of different cellular progresses such as transformation, differentiation, stimulations or stress. When they were first identified in the late 80s, they were considered cellular waste from cell damage or by-products of cell homeostasis. However, recent studies indicate that EVs are functional vesicles for intercellular communication by delivering a complex cargo of proteins, lipids, and nucleic acids to neighbor cells which allows programming of the cells and progressing in disease development [163, 164, 165]. Mainly, EVs are classified into three categories: exosomes, microvesicles, and apoptotic bodies [166]. Among them, exosomes are endosomal originated nanoscale EVs ranging from 30 nm to 100 nm in size [163, 167]. The structure of the exosomes is comprised of the lipid bilayer, transmembrane proteins, and a hydrophilic core containing proteins, mRNAs, and microRNAs (miRNA) in a spherical shape [163, 167]. They share some common components such as endosomal trafficking

proteins, membrane trafficking proteins, fusion proteins, gene expression proteins, heat shock proteins (HSP70, HSP90), signal transduction proteins, lipid rafts, and cytoskeletal proteins, tetraspanins (CD63, CD9, CD81, CD82), metabolic enzymes, adhesion molecules, nucleic acids, etc. (Figure 2.3). [163, 168].

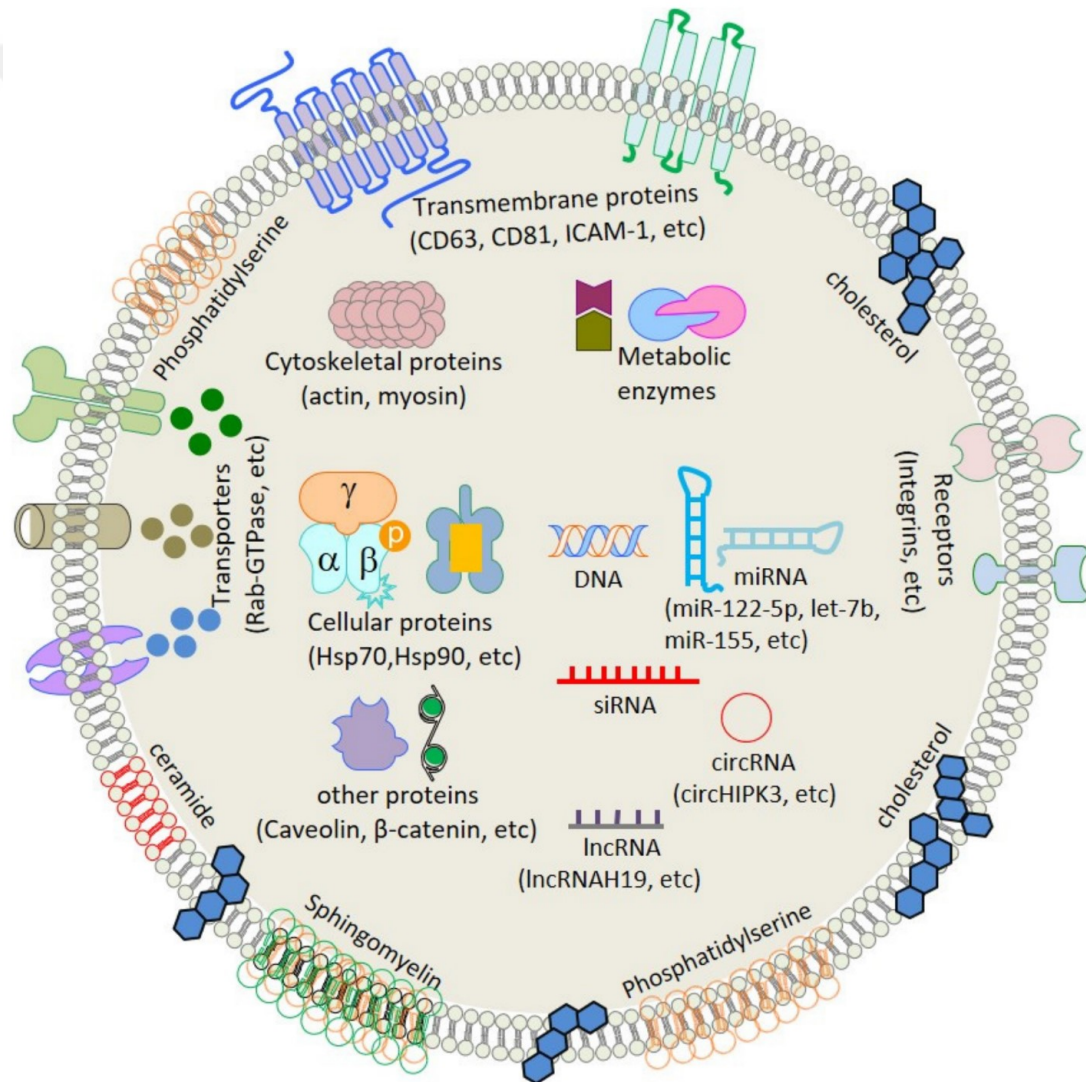


Figure 2.3: Illustration of exosome structure and functions. *Reproduced from [7] with permission from MDPI.*

In addition, recent research on cancer, cardiovascular, renal, and neurodegenerative diseases have showed the use of exosomes in diagnostics, prognostic and therapeutic purposes [166, 165]. Specifically, the role of exosomes in kidney diseases/disorders have been extensively investigated, and they have been considered as a potential biomarker for lupus nephritis (LN), AKI, diabetic nephropathy (DN), and also renal fibrosis and chronic kidney disease (CKD) progression, polycystic kidney disease (PKD), kidney transplantation, and renal cell carcinoma (RCC) [163, 167]. These vesicles are secreted from renal cells to urine that provides a very significant fashion for non-invasive sampling [168].

Exosomes can be isolated by bodily fluids such as blood, urine, saliva, breast milk, cerebrospinal and ascitic fluids, gastric juice, bile, sputum, bronchoalveolar lavage, semen, or tears. The physical properties (e.g., viscosity) and chemical composition (e.g., fat and protein content) can influence the isolation procedure and require adjustments in protocols [169]. While employing exosomes as a biomarker, major challenge is their high-yield, intact, and non-contaminated isolation. Various strategies have been investigated, and they are mainly divided into two categories: (i) conventional methods, which cover ultracentrifugation techniques, polymer precipitation (Poly-Ethylene Glycol (PEG)-Based Precipitation), size-based techniques (ultrafiltration, size exclusion chromatography), immunoaffinity chromatography. (ii) other techniques cover commercial products (e.g., exoEasy Maxi kit (QIAGEN), MagCapture™ Exosome Isolation Kit PS (Wako) and Minute™ Hi-Efficiency Exosome Precipitation Reagent (Invent)), microfluidic-based methods (e.g., inertial lift force, viscoelastic flow, deterministic lateral displacement (DLD), acoustic waves, dielectrophoretic and electrophoretic techniques) [166, 170, 171]. The isolation protocol varies depending on starting material, the required purity of isolates, and the availability of equipment [172]. Among them, differential ultracentrifugation is considered as the gold standard method that includes sequential centrifugation steps to enrich <200 nm of vesicles [172]. Briefly, the isolation generally starts at a low speed (e.g., 300 g for 10 min) to remove dead cells and bulky apoptotic debris. The second step employs higher speed rates (e.g., 1000 g to 20000 g) to remove larger vesicles and debris. Lastly, the highest speed (~100000 g) is applied for isolating EVs including exosomes.

For the characterization or further analysis, the pellet (which contains exosomes) is dissolved in PBS and stored at -80 °C. The enhancement pellet purity can be washed with PBS at 100000 g. The main drawbacks of ultracentrifugation are (i) time-consuming process ($\sim$ 4-5 h), (ii) low recovery, (iii) potential damages on exosome surface due to high centrifugal forces, and the need for high volumes ($\sim$ 15 mL[173]) [174]. All these challenges would hinder the quality and applicability for further analyses on exosomes such as proteomic and transcriptomic studies [169]. On the other hand, microfluidic-based methods hold some superior fashions such as low sample consumption, rapid separation, and higher separation yield [166].

2.3 Aim of This Study

In the scope of this study, we aim to develop cost-effective, and easily and large-scale manufacturable metasurfaces. To achieve this goal, off-the-shelf optical disks were recycled and turned into sensors substrate. To create a plasmonic sensing layer, we initially fabricate (i) Ti (adlayer)-Ag-Au and (ii) only Ag-coated metasurfaces. We then compare both bulk sensitivity and surface sensitivity on these sensors.

In order to improve the detection limits, we have also presented two different enhancement strategies, which include biomaterial integration and the decoration of secondary plasmonic layers through either binding or seeding of nanoparticles. The first strategy combines Poly-L-Lysine (PLL) material and AuNPs on the nanoperiodic structure. The second strategy facilitates the formation of NI on PLL-coated sensor through gold salt (Chloroauric acid (HAuCl_4)) and its reduction agent (hydroxylamine hydrochloride ($\text{HONH}_2\cdot\text{HCl}$)).

As a disease model, kidney diseases are considered to evaluate the performance of these sensors since they are prevalent in the society, and each step to minimize this problem would have great impact in the field. However, current biomarkers for kidney diseases limit the diagnostic procedure since they are not efficient

as elaborated above. In this regard, exosomes are chosen as a biomarker model in kidney diseases since recent incremental numbers of studies in that manner state their significance. Yet, isolating exosomes has also limitations with current generic methods. we therefore implement a microfluidic-based platform for exosome isolation that offers notable fashions like cost-effectiveness, facile-to-use, and easy manufacturing.

Overall, this thesis is constructed on five pillars: (i) fabricating a low-cost, easy-to-obtain, large-scale, and facile-use metasurface-based plasmonic sensor; (ii) enhancing the sensitivity of the metasurface-based plasmonic sensor with chemical-based strategies; (iii) surface functionalization that is specific to capture and detect exosomes ; (iv) developing a portable and easy-to-use optical system for quantitative measurements that even a layperson with minimum training can use it properly through user-friendly software; and (v) developing a filter-integrated microfluidic chip to isolate exosomes. Each step of the fabrication and biomarker (exosome) detection was characterized at the nanoscale levels employing Atomic Force Microscopy and Scanning Electron Microscopy. The chemical composition was characterized with X-Ray Photoelectron Spectroscopy analyses. Contact angle measurements were carried out to assess hydrophilicity/hydrophobicity of the sensors. In sum, we present a platform technology to detect minute concentrations of biomarkers over a wide-sensing area, and also resolve cost- and complexity-related fabrication challenges. This strategy and the developed system hold ground-breaking potential to shift our know-how for early diagnosis, as well as immediate treatment in the near future.

Chapter 3

Materials and Methods

3.1 Materials

Methanol (99.7%), Poly L Lysine (PLL), 3-Mercaptopropanyl-N-hydroxy-succinamide ester (3-MNHS), Sodium citrate dihydrate, Gold (III)chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), biotin and hydroxylamine hydrochloride were purchased from Sigma-Aldrich. Absolute ethanol (99.9%) and Glycerol (85%) were purchased from Isolab Chemicals. Phosphate Saline Buffer (PBS) tablets were obtained from Biomatik. The other organic solvents including ethanol (96%, Soltek), isopropanol alcohol (99.5%, Lobal Chemie), and acetone (99.5%, Birpa) were purchased from the companies stated here. Optical DVD discs (HP) were used as plastic surfaces (PS). Avidin was purchased from Thermo scientific. Gold (999.9) and silver (999.0) were purchased from the Istanbul Gold Refinery. All chemicals were used without further purification, and deionized water was used throughout. In cell culture studies, penicillin/streptomycin, L-Glutamine, DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, Biowest), and Trypsin-EDTA were purchased from Gibco. Fetal Bovine Serum (FBS) was purchased from Biowest.

3.2 Plastic Template Surface Preparation

The nanoperiodic substrate of the sensor was attained from digital versatile discs (DVDs) [29] since they are inexpensive, easily accessible, and commercially available products for extensive usage. Multi-layers of the DVDs (top plastic surface, metal-coating layer, photoresist, nano periodic surface grating, and polycarbonate-plastic layer), were removed as stated in the literature [34]. Briefly, (i) the top coating plastic surface was removed from the optical discs physically with a knife. If metal-coating has remained, air pressure can be applied. (ii) The dye and photoresist layer was removed by a mixture of ethanol (EtOH) and methanol (MetOH) (1:1: v:v). (iii) Lastly, the distance between periodic structures (e.g., height, width) was adjusted with the mixture of acetone and isopropanol (1:4; v:v) for 60 s. After (ii) and (iii) steps, the surface was washed out. After completing all these steps, metasurface was fabricated by coating 10 nm of Ti, 30 nm of Ag, and 15 nm of Au on the grating side of the plastic substrate. This coating process was executed inside a thermal evaporator by fixing the substrate to a vacuum coating chamber with a pressure level of 2×10^{-6} Torr. Once produced, the consequential sensor was cut into 1.5 cm x 1.5 cm for further studies. For the silver top surface, a prepared optical disk coated with 65 nm of Ag with thermal evaporation with a similar protocol. Once the metamaterial was fabricated, the surfaces were divided into 1.5 cm x 1.5 cm for further studies.

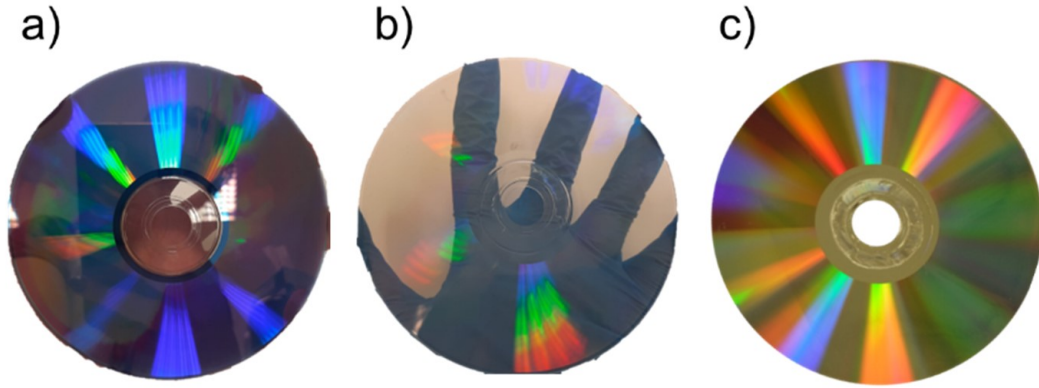


Figure 3.1: Recycling optical disks to the sensor surfaces. (a) A commercially available optical disk is presented. (b) Cleaned optical disks are ready for the metal coating. (c) A gold-coated optical disk (a metamaterial sensor) is presented.

3.3 Biomaterial Modification

After the sensors were fabricated, they were cleaned with 5 minutes sonication in ethanol and dried with the compressed air. Later, 10 mg/mL of PLL stock solutions were prepared in water. Followingly, the stock solution was diluted to 0.05, 0.01, 0.5, and 1 mg/mL with PBS to find the optimum PLL concentration. The cleaned sensor surface was dipped into 0.05, 0.01, 0.5, and 1 mg/mL of PLL solution and incubated overnight at +4 °C. When the incubation was concluded, the surface was washed with PBS and dried with the compressed air for the next steps, i.e., the AuNPs integration and NI construction. .

3.4 Synthesis of Gold Nanoparticles (AuNPs)

To synthesize gold nanoparticles (AuNPs), the seeding reaction were executed. By the citrate reduction, the seeding solutions were first prepared. For the citrate reduction, 90 mL of sodium citrate solution was prepared by adding 60 mg of

sodium citrate dihydrate to distilled water and boiled for 10 minutes under vigorous stirring. Afterwards, 6 mg of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ were applied to the solution and kept boiling for another 30 minutes. At the end of boiling for 30 minutes, the solution was allowed to cool down to $90\text{ }^\circ\text{C}$. When the temperature was reduced to $90\text{ }^\circ\text{C}$, 25 mM of sodium citrate (3 mL) and 1 mM of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ solution (3 mL) were introduced to the seeding solution, and then, started to boil for 30 minutes. At final, the synthesized particles were cooled down and stored at $+4\text{ }^\circ\text{C}$ for the further use.

3.5 Integration of AuNPs to the Sensors

After the coating of sensors with PLL, the sensors were then dipped into AuNPs-containing solution. To preserve nanoporous structures, the number of integrated AuNPs needs to be optimized. Therefore, the stock solution of AuNPs and the serial diluted samples (1:2, 1:5, 1:10, 1:20 and 1:50) were tested on 0.5 mg/mL of PLL-modified sensor and incubated at $+4\text{ }^\circ\text{C}$ overnight. Afterward, the sensors were washed with water, dried with the compressed air, and then, assembled with the microfluidic module for sampling.

3.6 Nanoisland Formation

After the surfaces were modified with PLL, NI formation was performed by dipping into the seeding solution (HAuCl_4 and $\text{HONH}_2\cdot\text{HCl}$). At first, 1 mM of HAuCl_4 was prepared in distilled water. Similarly, 1 mM of $\text{HONH}_2\cdot\text{HCl}$ was prepared as a stock solution. The seeding solution was prepared by a mixture of the equal concentration and volume of seeding solution (HAuCl_4 and $\text{HONH}_2\cdot\text{HCl}$) (1:1: v:v). To find optimum seeding concentration and time, 10, 20, 50, and 100 μM of HAuCl_4 and $\text{HONH}_2\cdot\text{HCl}$ mixture were assessed while keeping PLL concentration (0.5 mg/mL). The PLL-modified surfaces were dipped into each

concentration of the seeding solution for 5 minutes. Once the process was completed, the sensor surfaces were washed with distilled water and dried. Once the surfaces were assembled with microfluidic modules, they were ready-to-use for further experiments (bulk sensitivity measurements and biomarker detection).

3.7 Preparation of the Microfluidic

Microfluidic modules were integrated for introducing samples to the sensor surface. The design of the module contained two channels made of three layers: (i) polymethyl methacrylate (PMMA, 2 mm of thickness), (ii) double-sided adhesive film (DSA, 50 μm of thickness), and (iii) sensor layer. The PMMA component had inlet and outlet ports (0.67 mm) on top of the channels, and the tubings (0.32 mm inner diameter, 0.76 mm outer diameter, and Polytetrafluoroethylene (PTFE), Adtech) were connected here for sampling. The microfluidic channels were created with a DSA layer (Figure 3.2), which was placed between PMMA and sensor layer. PMMA and DSA layers are designed using RDWorks and cut by a laser cutter instrument (LazerFix, Turkey)[175, 176, 177] (Figure 3.2). The fabrication of the microfluidic modules was started by cleaning PMMA layers with ethanol, and any potential dusts were removed by the compressed air. The first layer of the DSA was then removed and stuck on the PMMA. Next, ~ 15 cm of tubing was cut and fixed to the inlet and outlet holes using epoxy (Pattex). Once the microfluidic module was fabricated, the second layer of the DSA was removed and assembled with the sensor. For sampling, the tubings were connected to a syringe pump system.

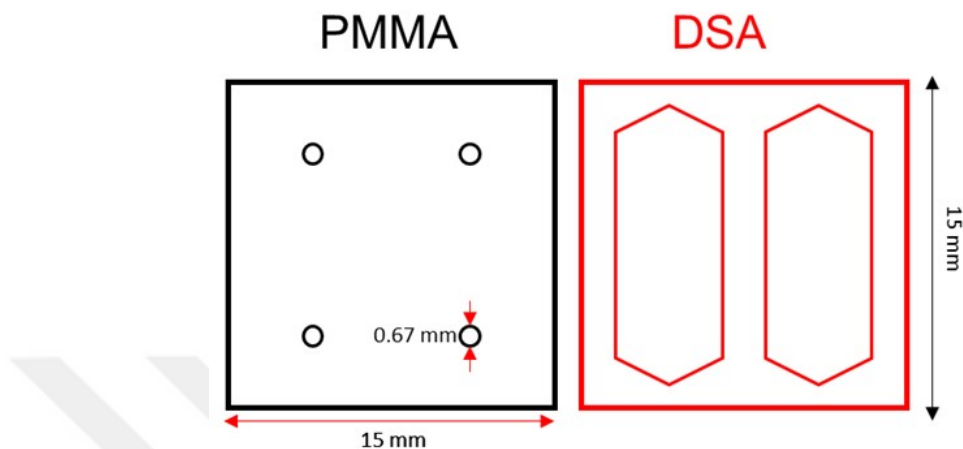


Figure 3.2: A schematic depicts PMMA and DSA layers and their designed dimensions through RDworks software.

3.8 Microfluidic Assembly with Sensor

The fabricated microfluidic modules were assembled with the sensor surfaces by peeling the second layer of DSA film (Figure 3.3b). After the assembly, the microfluidic module was connected to a syringe pump to achieve sample flow through the channels. The flow rate was set to 10 $\mu\text{L}/\text{min}$ for sampling and washing steps (Figure 3.3c).

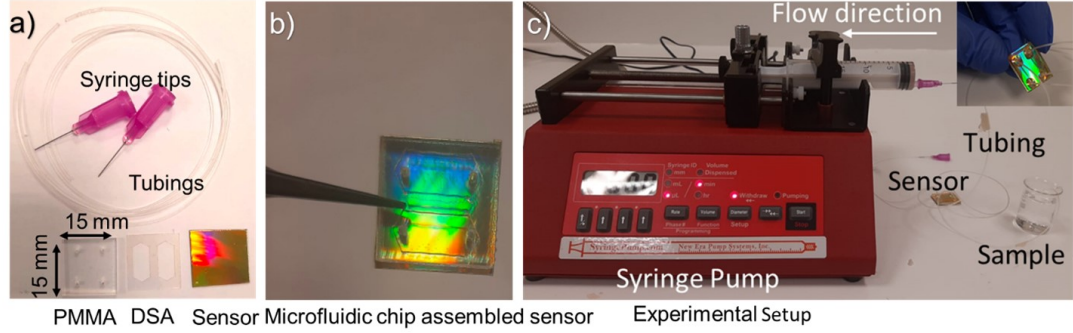


Figure 3.3: The components of microfluidic chip and the photographs of experimental setup are presented. (a) Components of the microfluidic chip and sensor is exhibited. (b) Microfluidic chip is assembled with a sensor, and (c) experimental setup is demonstrated.

3.9 Portable Optical Setup

A portable system with optical elements was developed for spectroscopic measurements on the sensor surface. There are three main components: (i) portable CCD spectrometer (Thorlabs, CCS175/M) scanning from 500 nm to 1100 nm, (ii) a light source in the visible range (400–1300 nm operating range), and (iii) optical components (cosine corrector, lenses, linear polarizer, beam splitter, illuminator input, iris) (Figure 3.4). In the measurement platform, the light is illuminated from the source (number 8 in Figure 3.4d); it passes through the lens (number 7 in Figure 3.4d) and reach to the linear polarizer (510–800 nm) (number 6 in Figure 3.4d). Afterward, the light passes to the (400–700 nm) optical aperture (number 5 in Figure 3.4d) and continues until reaching the beam splitter (50:50) (number 3 in Figure 3.4d). Later, 50% of the light passes the second lens (right on the sensor place, number 4 in Figure 3.4d), and it is then reflected from the sensor surface. Again, the light passes through the second lens, the beam splitter, the 3rd lens (number 2 in Figure 3.4d), and finally reaches the spectrometer (500 – 1000 nm, FWHM <0.6 nm) with a cosine corrector-integrated fiber cable (number 1 in Figure 3.4d). As a result, the optical intensity (a.u.) and wavelength (nm) of the reflected light are analyzed via Thorlabs OSA software.

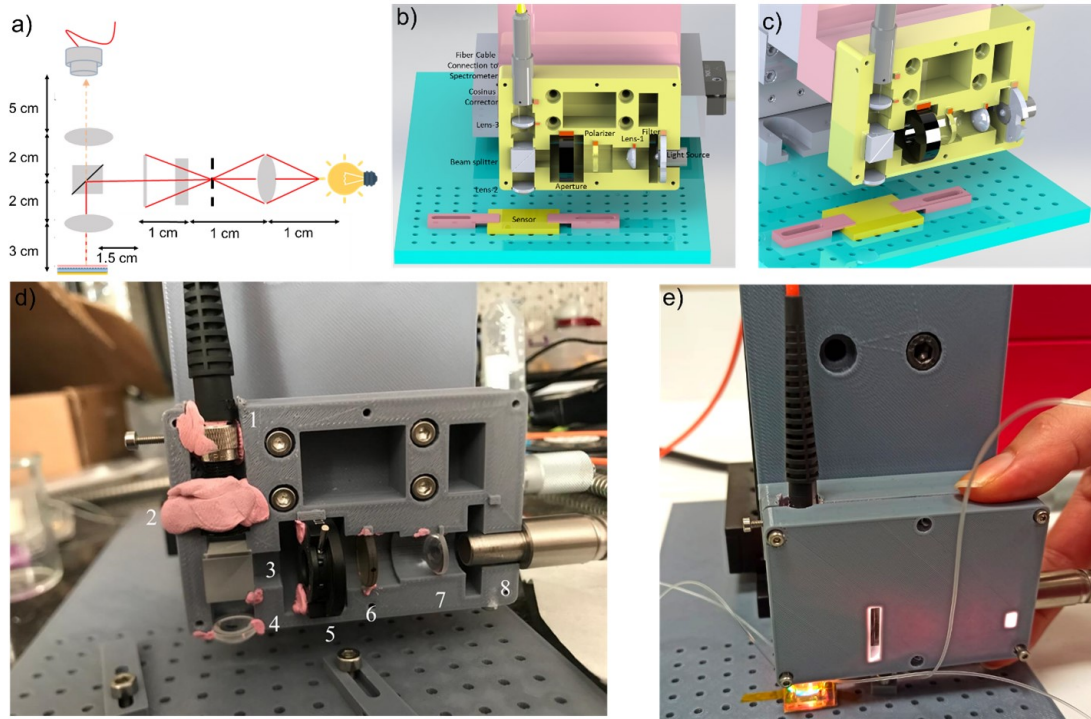


Figure 3.4: (a) A schematic illustrates the optical path in the set-up. (b-c) The 3D design of the optical setup is presented. (d-e) The photographs of portable platform are shown.

3.10 Real-Time Data Monitoring *via* In-House Interface

The spectrometer comes with a commercial software called Thorlabs OSA software, which only presents end measurements, and it does not enable to monitor the data in real-time. This limits to monitor the binding of analytes. Although the resonance of these plasmonic sensor is majorly located between 550 nm and 650 nm of wavelength, the commercial software scans a broad wavelength (500 nm – 1100 nm), requiring more computer power for measurement. In this regard, an in-house interface was developed by writing a code on MATLAB GUI to achieve a user-friendly environment and to minimize user interpretation and

training. Briefly, the code directly collects the data from the spectrophotometer and reduces the noise by polynomial fitting (5th degree) after determining spectral peaks. The sensor resonance points took place from 550 nm to 650 nm of wavelength range, which were then used for the 5th degree of the polynomial fitting. After the fitting, the MATLAB peak finder determined the resonance points in real-time and plotted them simultaneously. Figure 3.5 indicated the code interface composed of multiple windows: (i) Main, (ii) Options, and (ii) Incoming Data. The Main window includes “Start,” “Save,” and “Reset” buttons to activate and manage the software. “RT Data Viewing Interval” section controls the y-axis (Wavelength) of the Real-Time Data graph. The fitting interval can be controlled “Fitting Wavelength Interval” section. In the “Option” window, the fitting degree and resonance type (dip or peak) can be adjusted. The third window displayed data from the spectrometer and background save. Finally, a portable optical device that could measure plasmonic signals over time was achieved.

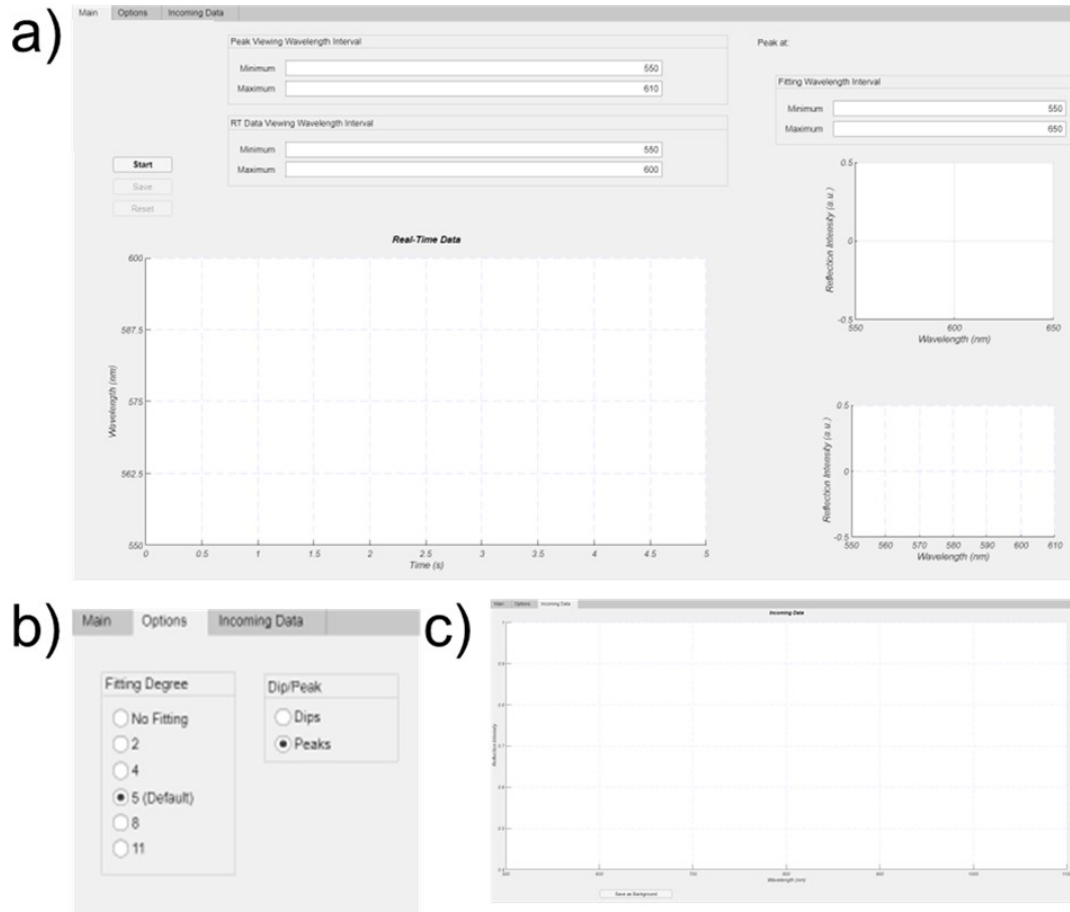


Figure 3.5: In-house software is developed through MATLAB GUI. (a-c) Different sections/windows of the software are presented.

3.11 Bulk Sensitivity Measurement with Glycerol

Once the sensors were fabricated, the bulk sensitivity was benchmarked for two types of sensors (gold and silver sensors) by applying various glycerol solutions (1% to 70%). This strategy leads to create a change in RI on the sensor without any binding. For this respect, the stock solution of glycerol was diluted with distilled water. The measurements were performed in both MATLAB

GUI-based interface (real-time measurements) and Thorlabs OSA software (end measurements). Before the measurements, the background was recorded using the mirror, thereby collecting all reflected light. Afterward, distilled water (0% of glycerol) was introduced into the channels with 10 μL /minutes of flow rate in order to create a baseline. The glycerol solutions were then applied to the channels starting from less concentrations to higher ones. As the MATLAB GUI-based interface provides real-time data, we collected a ladder-type data in this analysis.

3.12 Surface Functionalization with Anti-CD81 Antibody

Thiol chemistry is a commonly applied compound for gold and silver surfaces. In this regard, 10 mM of 11-MUA (dissolved in ethanol) was employed since it contains a thiol group at one end and a carboxyl group at another end. The thiol group creates a dative bond and presents carboxylic groups. The bare silver and gold top surfaces were incubated in 11-MUA solutions overnight at room temperature and in a dark environment. After the incubation, the surfaces were washed with ethanol and assembled with microfluidic modules. A mixture of EDC/NHS (100 mM:50 mM) (prepared in MES, pH 6.0) was applied on the 11-MUA modified surface for an hour at room temperature to form succinimide groups, allowing to bind protein anchoring molecules such as protein G that help to immobilize antibodies in their proper orientations through the Fc portion of antibodies. After creating succinimide groups, 100 $\mu\text{g}/\text{mL}$ of protein G (prepared in PBS) was applied to the channels. Later, 50 $\mu\text{g}/\text{mL}$ of anti-CD81 antibodies—which have an affinity to exosome tetraspanin proteins, were applied on protein G immobilized surface for 4 hours at +4 °C. AuNPs-integrated and NI-formed surface directly functionalized with 100 $\mu\text{g}/\text{mL}$ protein G (in PBS). Then 50 $\mu\text{g}/\text{mL}$ of anti-CD81 antibodies were immobilized by incubating for 4 hours at +4 °C. The same protocol was performed for immobilizing anti-CD63 antibodies.

3.13 Microfluidic Chip Fabrication for Exosome Isolation

Similar to the module fabrication, a microfluidic chip was manufactured by the laser cutter system (LazerFix) using RDWorks V8 software. The chip isolates exosomes through two filter membranes (200 nm and 50 nm of pore size). Briefly, the chip contains five layers of PMMA (2 mm of thickness) that are connected with DSA films (50 μm of thickness) (Figure 3.6a). The first three layers of PMMA were located at the top of the filter membranes. Considering the diameters of exosomes (40-120 nm) [178], two filter membranes with distinct pore diameters were employed on the chip. Therefore, the first filter with 200 nm of pore diameters eliminated large debris, whereas the second filter with 50 nm of pore diameters enabled the isolation of exosomes. The order of assembly is shown in Figure 3.6b. After the assembly, tubings were connected to the inlet and outlet ports (Figure 3.6c-d).

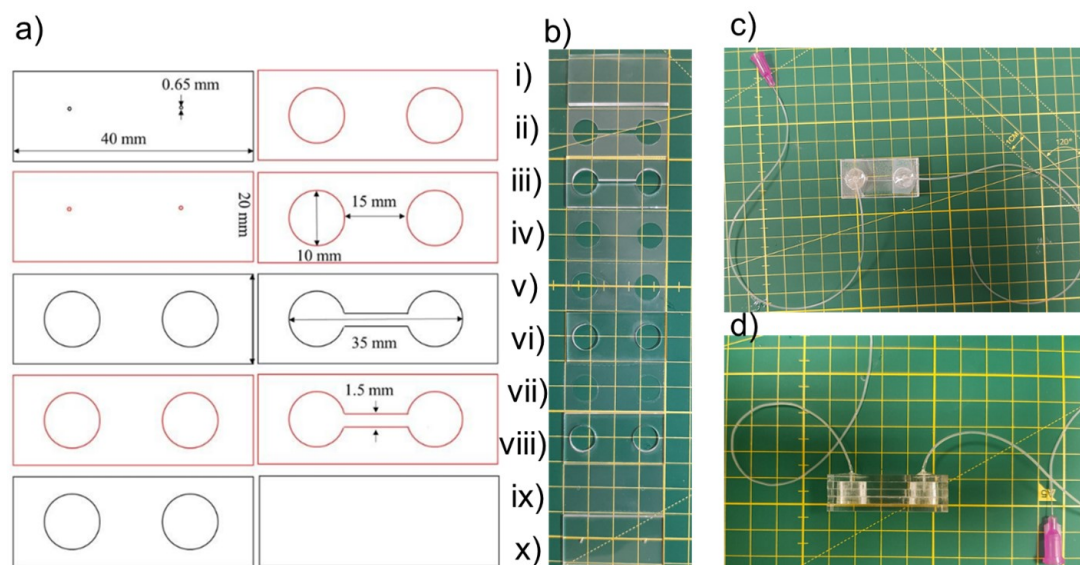


Figure 3.6: (a) The design of a microfluidic chip for exosome isolation (b) The assembly order and (c-d) top- and side-view of the microfluidic chip are shown. *Reproduced from [8] with permission from American Chemical Society.*

3.14 Cell Culture Protocols

Human Embryonic Kidney (HEK293) cells were grown in DMEM/F-12. Penicillin/streptomycin (1% and 5 mL), 1% of L-Glutamine (5 mL) and 10% of Fetal Bovine Serum (FBS) (50 mL) were added into the medium as supplements for cell growth. Before passaging cells, the medium and 0.25% of Trypsin-EDTA were kept in a water bath at 37 °C. Before the first use, the hood was sterilized with UV for 15 minutes. Followingly, the working area in the hood was wiped with 70% of ethanol. Cell confluency was investigated with a light microscope (Zeiss Inverted Fluorescence Microscope). Afterward, the cell flasks were placed onto the hood, and the medium in the cell flask was drawn and discarded using a pipettor. Cells were washed with 2 mL of PBS twice. To detach the cells, 0.25% Trypsin-EDTA (1 mL) was introduced onto the cells and incubated for 5 minutes at 37 °C. After 5 minutes, 4 mL of medium was applied to the flask to neutralize the Trypsin-EDTA effect over the cells. Finally, we added 4 mL of fresh medium to 1 mL of cell from the flask (1:5 dilution), and the cells were then transferred into new flasks, which were placed in the incubator at 37 °C under a 5% of CO₂ atmosphere.

3.15 Exosome Isolation

The exosomes were isolated HEK293 cell line. When the cells were confluent, FBS free medium was introduced to cells and incubated for 4 days. The medium was collected from the cells to prepare for the exosome isolation. The first step was to remove residual cell debris and other molecules from the collected medium. For this purpose, the medium was added to the Eppendorf tubes and centrifuged at 20,000 g (14,000 rpm) for 15 minutes at room temperature. When the centrifugation was over, the supernatant was taken and transferred to a new falcon tube since exosome are present in the phase of supernatant. Before introducing the sample to the microfluidic chip, the supernatant was filtered with 0.22 μ m syringe filter, and it was then supplied to the microfluidic chip. On the course

of exosome isolation, the flow rate was set to 40 $\mu\text{L}/\text{minutes}$, and exosomes were isolated from the outlet port.

3.16 Sampling and Measurements on the Optical Device

Before the capture experiments, exosomes were quantified with NTA integrating a 488 nm blue laser for visualizing the particles. The microfluidic chamber on the NTA system has screws on top to prevent leakage. Before applying the sample, screws were tightened, and followingly, distilled water (filtered) was introduced to remove any potential residues. After a clean view was obtained on the screen, the number of capture and duration was set to 5 and 60 seconds; dilution rate was entered accordingly; viscosity was set to 0.92 c.Pa; and temperature was kept at 25 °C. When all parameters were entered to the system, ~ 0.5 mL of the isolated samples were introduced to the chamber, and measurements were initiated. Once the measurement was completed, the threshold was designated, and the information of particle size and concentration was quantified by the system automatically.

Later, the stock solution of exosomes was diluted to 10^8 particles/mL with PBS. The plasmonic measurements were monitored on the portable optical setup. Initially, we created a baseline with PBS within 500 seconds. Then, 10^8 particles/mL of exosome solution was applied for 45 minutes at 10 $\mu\text{L}/\text{minutes}$ of flow rate. To remove unbound particles, the sensor was washed out with PBS for 5 minutes at the same flow rate.

In addition to exosome spiked in PBS, we aim to imitate pre-clinical samples by spiking exosome into artificial urine since urine samples are collected from patients with kidney diseases. The aforementioned experimental procedure was followed for the detection of exosomes spiked in artificial urine.

3.17 Western Blot

Western Blot analysis was performed by targeting tetraspanins proteins (CD63 and CD81) on exosomes. The stock sample (5×10^9 particles/mL) to load onto gel made of 10% of resolving gel (1.5 M Tris (pH 8.8), 10% SDS, 30% Acrylamide mix, 10% APS and TEMED) and 5% of stacking gel (0.5 M Tris (pH 6.8), 10% SDS, 30% Acrylamide mix, 10% APS and TEMED). To load samples into the wells, exosome samples were mixed with loading dye and 1 M of DTT in 1:1 (v:v) ratio. After the samples were loaded to the wells, the voltage was set to 90 V for 30 min, then 120V for 45 minutes in the presence of a running buffer. The running buffer was prepared with 25 mM of Tris, 192 mM of glycine, and 10% of SDS (v:v) (pH was adjusted to pH 8.3). After the running process was completed, proteins in the gel were subsequently transferred to nitrocellulose membranes by semi-dry blotting with 25V 1.3 ampere for 40 minutes. The blocking was performed with 5% of milk in TBST buffer at room temperature for an hour on gentle shaking. Later on, anti-CD63 antibodies (ThermoFisher, MA5-30187) and anti-CD81 antibodies (Sigma-Aldrich, SAB3500454) were diluted from their stock (1 mg/mL) at a 1:1000 dilution rate with the 5% of milk and applied on the membrane and incubated overnight (18 h) at a $+4^{\circ}\text{C}$ on gentle shaking. At the end of the incubation of anti-CD63 antibodies and anti-CD81 antibodies, the membranes were washed three times for 10 min with TBST. After the washing step, the membranes were incubated with HRP-conjugated secondary goat anti-rabbit antibody for 1 hour at room temperature on gentle shaking. At the end of incubation, the blots were washed 3 times for 10 min with TBST and the chemiluminescent reagent (Thermo Fisher Scientific Inc.) was then applied to blots for 5 minutes at room temperature under dark environment. The images were obtained from a Bio Rad Chemidoc XRS Plus system.

Chapter 4

Characterization Methods

In this study, the characterization methods were categorized into two sections: (i) surface characterization and (ii) chemical characterization. Scanning Electron Microscope (SEM) and Atomic Force Microscope (AFM) were used to determine periodicity, AuNP integration, and the formation of NI on the surfaces. SEM was also used for the size characterization of exosomes. The transmission Electron Microscope (TEM) and Zetasizer were used to determine AuNP size as well. The NTA was utilized for calculating the size and concentrations of exosomes. To functionalize the sensor surface, several chemical processes were carried out, and therefore, contact angle and X-Ray Photoelectron Spectroscopy (XPS) were executed for this manner.

4.1 Surface and Size Characterization

4.1.1 Scanning Electron Microscope (SEM)

Manfred Von Ardenne invented the SEM in 1937 in Berlin. Briefly, SEM utilizes a high-energy electrons in a focused beam to produce various signals at the samples surface [179], and focused electron beams scans over a surface to create

an image. When the electrons in the beam interact with the sample, the topography the composition of a specimen are obtained. Commonly, SEM instruments composed of the electron column, sample chamber, EDS detector, electronic console, and visual display monitor.

Herein, the nanoperiodic structure of the gold, silver-coated sensor surface, AuNPs-integration to the sensors, and NI formation on the nanoperiodic structures were observed through topographical analysis of the SEM (FEI Quanta 200 FEG). The gold and silver-coated surface, AuNPs-integrated, and NI-formed surfaces were investigated without coating since they have conductive layers.

As a result, the nanoperiodic structure of sensor surface was clearly visible in the Figure 4.1a for gold top and Figure 4.2a for silver top metasurfaces. Integration of AuNPs to the nanoperiodic structures was also successful (Figure 4.1b and Figure 4.2b). Similarly, NI was effectively constructed on the nanoperiodic structure for both gold and silver top metasurfaces (Figure 4.1c and Figure 4.2c.).

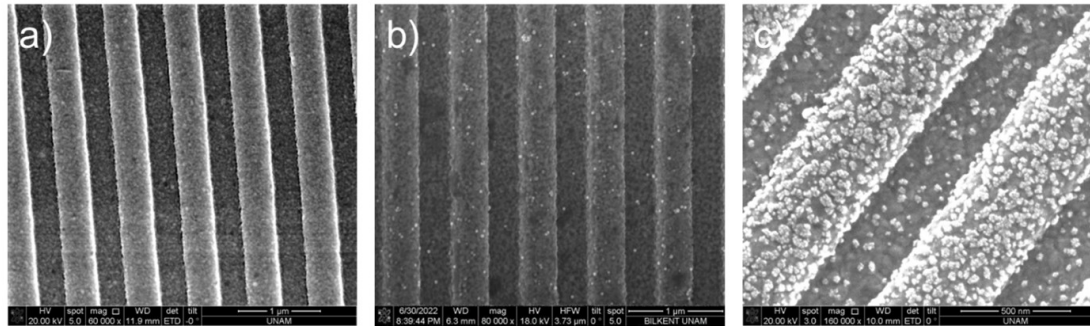


Figure 4.1: Surface characterization of the gold top metasurfaces. (a) Bare gold top surface. (b) AuNPs-integrated gold top metasurface. (c) NI-formed gold top metasurface.

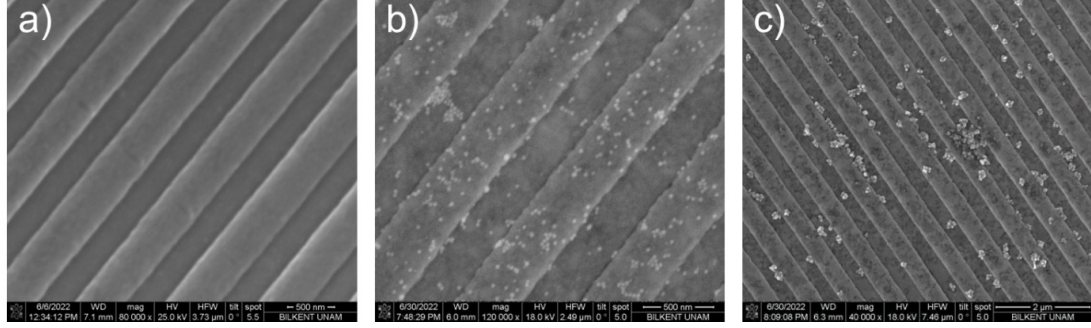


Figure 4.2: Surface characterization of the silver top metasurfaces. (a) Bare silver top surface. (b) AuNPs-integrated silver top metasurface. (c) NI-formed silver top metasurface.

Besides nanograting characterization of the sensor surface, the isolated exosomes were also imaged with SEM to determine their size and morphology. The samples were prepared by mixing with a 2% of paraformaldehyde (PFA) in 1:1 (v:v) ratio. We dropped on a cleaned silicon wafer (5 minutes of sonication in acetone, ethanol, and water, respectively) and left to dry on the wafer. Following, the exosomes were washed with ethanol three times to dehydrate. The samples were coated with 5 nm of Au/Pd and placed on the sample holder for the imaging studies with SEM (FEI Quanta 200 FEG) (Figure 4.3). The images were processed with Image J, and particle size was analyzed. The average particle size was determined as 87.25 ± 15.46 nm. The biggest particle size was 114.48 nm, and the smallest particle was 52.75 nm as depicted in Figure 4.3.

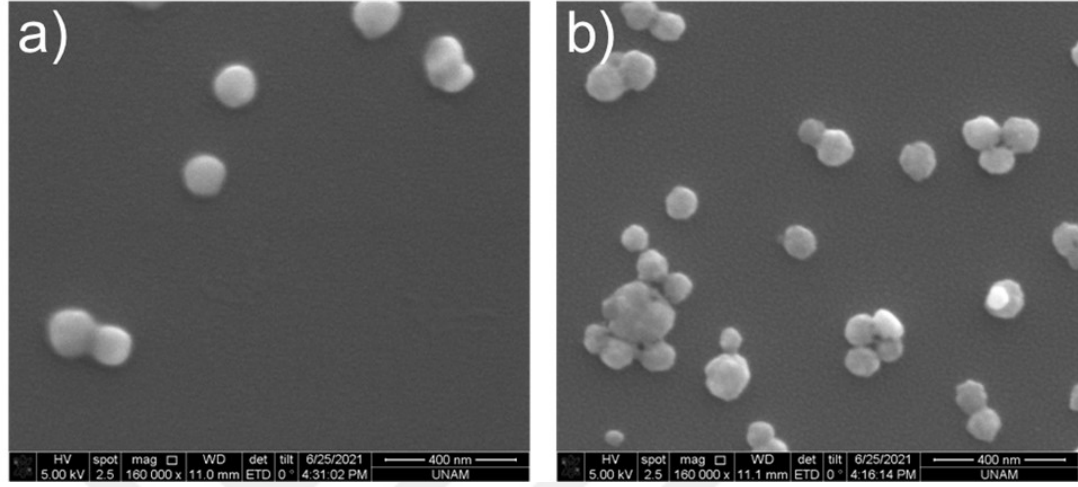


Figure 4.3: The SEM image of the isolated exosomes.

After the isolation, they were captured on the metamaterials-based plasmonic sensors decorated through anti-CD81 antibodies. Afterward, the captured exosomes on the bare gold top sensor surface were imaged by SEM, and the uncaptured surface was used as a control. As clearly seen in the SEM images (in Figure 4.4), the exosome was successfully captured on the sensor. It seems that they were adhered to the surface (both the space and hill side of the nanoperiodic structure), and this might be due to the incubation of exosomes and PFA treatment for the fixing process.

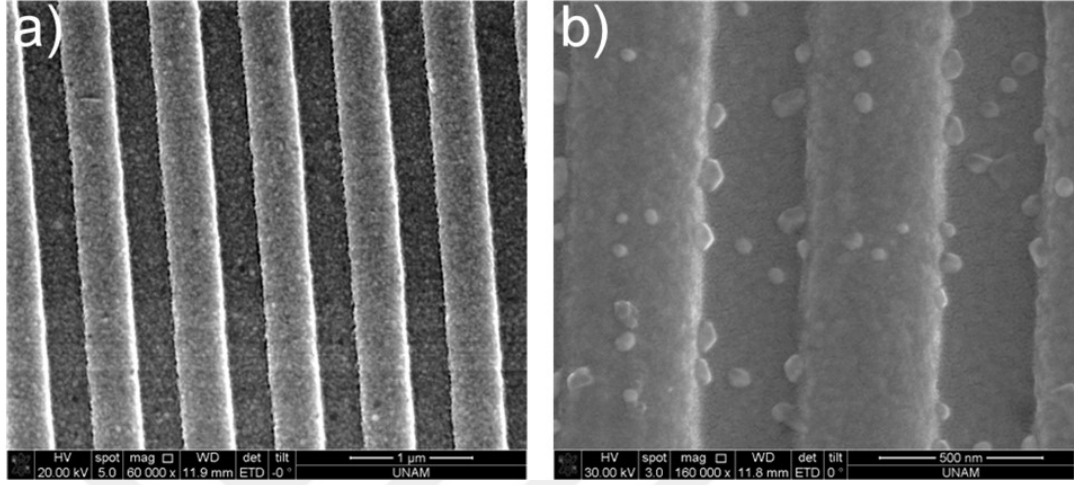


Figure 4.4: The SEM images of (a) the bare gold top metasurface and (b) the captured exosomes on the bare gold top modified with anti-CD81 antibodies.

4.1.2 Atomic Force Microscope (AFM)

AFM is one of the foremost characterization methods to image, measure and manipulate samples surface at the nanoscale. It is invented in 1985 by IBM scientists. Its principle relies on the cantilever deflection due to repulsive forces between atoms on the sample surface and atoms at the cantilever tip. The laser beam determines deflection [179].

In this study, the nanoperiodic structure of the gold and silver-coated sensor surface, AuNPs-integrated, and NI-formed on the nanoperiodic structure topographies were investigated with AFM instrument (Asylum, Oxford Instrument, UK) by using Nanosensors PPP – NCHR tip with 42 N m^{-1} force constant on AC Air Topography (Figure 4.5). We quantitatively measured height, period, and width of the nanoperiod structures of sensor. The imaging process was applied with a 0.6 Hz scanning rate, and the images were analyzed using Gwyddion (There were no data manipulation such as filters or smoothing). The images were obtained from the $2 \mu\text{m} \times 2 \mu\text{m}$ area.

The nanoperiodic grating structure and NI were observed explicitly (Figure 4.5). Per the AFM results, the sensor includes nanostructures with ~ 500 nm of width and ~ 740 nm of periodicity (Figure 4.5). The heights of the unmodified surfaces were low compared to AuNPs-integrated and NI-formed surfaces. Briefly, Figure 4.5a showed the topography of bare gold top surface with ~ 500 nm of width and ~ 740 nm of periodicity. The height was ~ 157 nm for the bare gold top surface, ~ 238 nm for AuNPs integrated gold top, and ~ 240 nm for the NI-formed surface. As clearly seen in the AFM profiles (Figure 4.5b-c), the AuNPs and NI were successfully constructed on the sensor.

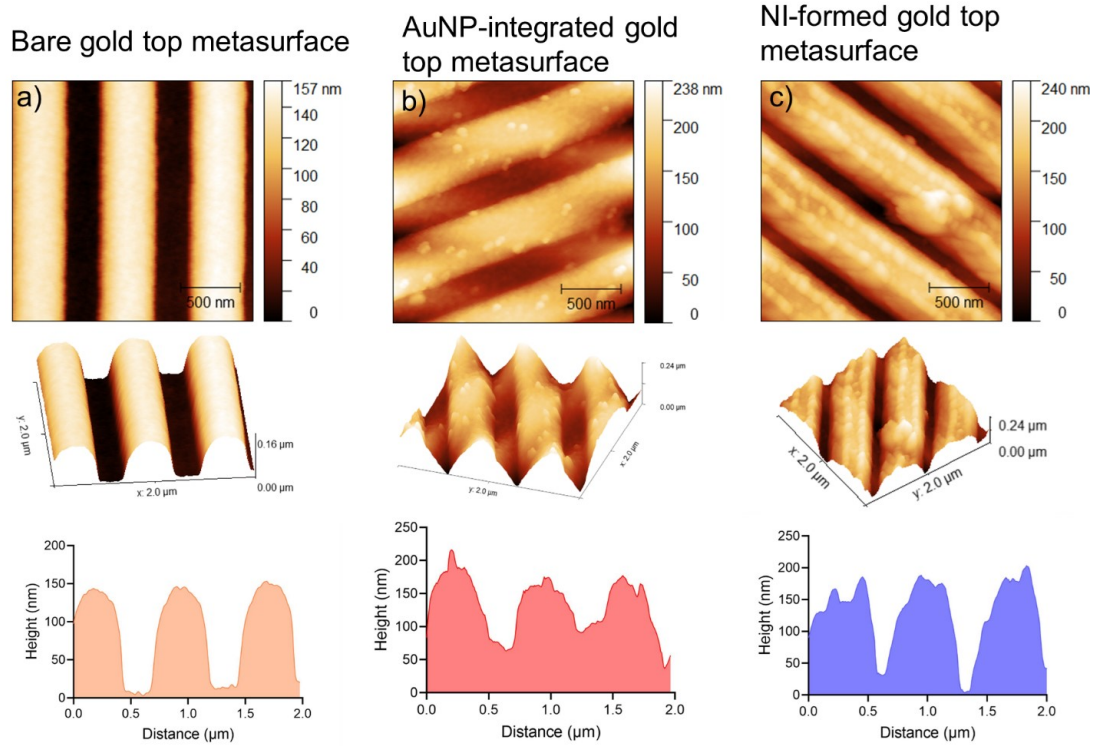


Figure 4.5: The 2D and 3D AFM images and the profile graphs of (a) the bare gold top, (b) AuNPs-integrated gold top, and (c) NI-formed gold top metasurfaces.

The nanoperiodic structure of silver top surfaces (bare, AuNP-integrated, and NI-formed) was observed explicitly (Figure 4.6). Per the AFM results, the sensor includes nanostructures with ~ 500 nm of width and ~ 740 nm of periodicity, similar to the gold top surface (Figure 4.6). The height values of the unmodified

surfaces were low compared to AuNPs-integrated and NI-formed surfaces. Briefly, Figure 4.6a shows bare gold top surface topography with ~ 500 nm of width and ~ 740 nm of periodicity. The height was ~ 184 nm for the bare gold top surface, ~ 201 nm for AuNPs integrated gold top, and ~ 202 nm for the NI-formed surface. As plainly seen in the AFM profiles (in Figure 4.6b-c), the AuNPs and NI were constructed successfully on the sensor.

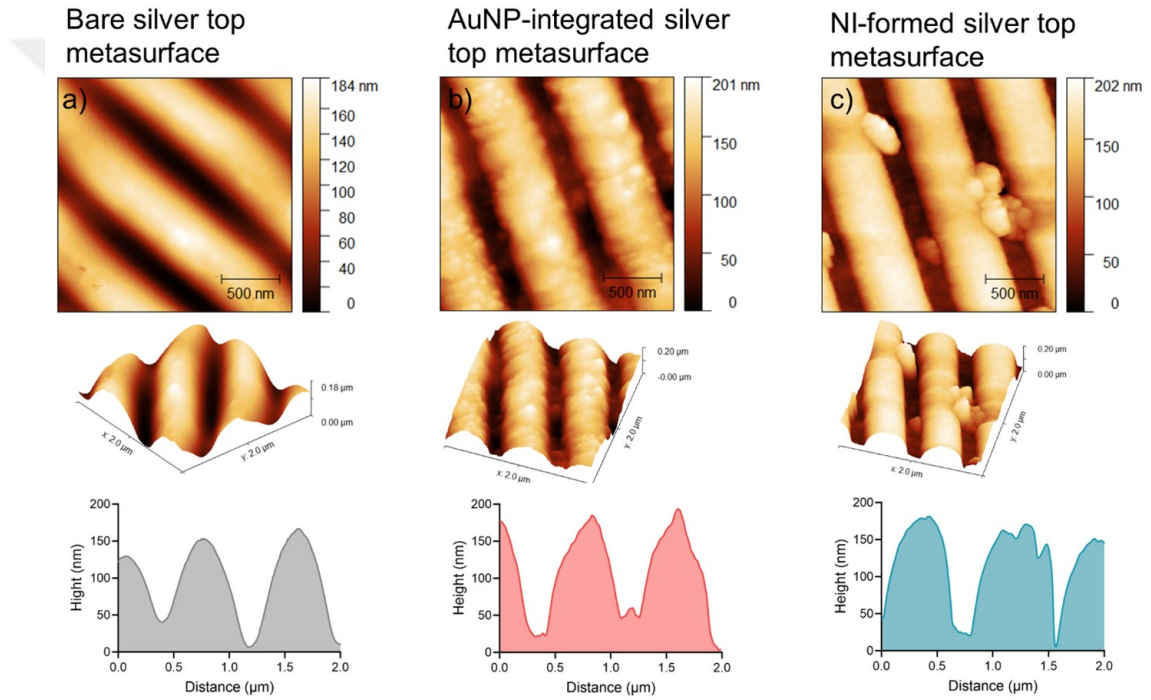


Figure 4.6: The 2D and 3D AFM images and the profile graphs of (a) the bare silver top, (b) AuNPs-integrated silver top, and (c) NI-formed silver top metasurfaces.

4.1.3 Transmission Electron Microscope (TEM)

Ernst Ruska and Max Knoll constructed the first TEM in 1931 at the University of Berlin. Ruska was then awarded the Nobel Prize for Physics in 1986 for this invention. The principle of the TEM is similar to the light microscope. However, a light microscope uses light in the visible range, and TEM utilizes electrons rather than light. The electrons travel from the electron gun to the

phosphor screen through the sample. In addition, for a proper TEM operation, the samples need to be ~ 100 -nm thick. The samples are then placed on TEM grids containing a metal frame and carbon (C)-based support film for imaging [179].

In this study, TEM analysis (FEI, Tecnai G2 F30, 300 kV) with energy-dispersive X-ray (EDX) spectroscopy) was performed to determine the shape and size of the synthesized AuNPs. Carbon-based grids were used for sample preparation. Synthesized particles were basically dropped on the TEM grid ($2\ \mu\text{L}$), left to dry, and imaged. Figure 4.7 indicated spherical AuNPs in the size of ~ 25 nm. The particle size was further calculated by the Image J software. The mean of the particle size was determined as 25.59 ± 6.27 nm. The largest particle diameter was 29.67 nm, and the smallest particle diameter was 20.17 nm. These values were obtained by taking into account 20 separate particles.

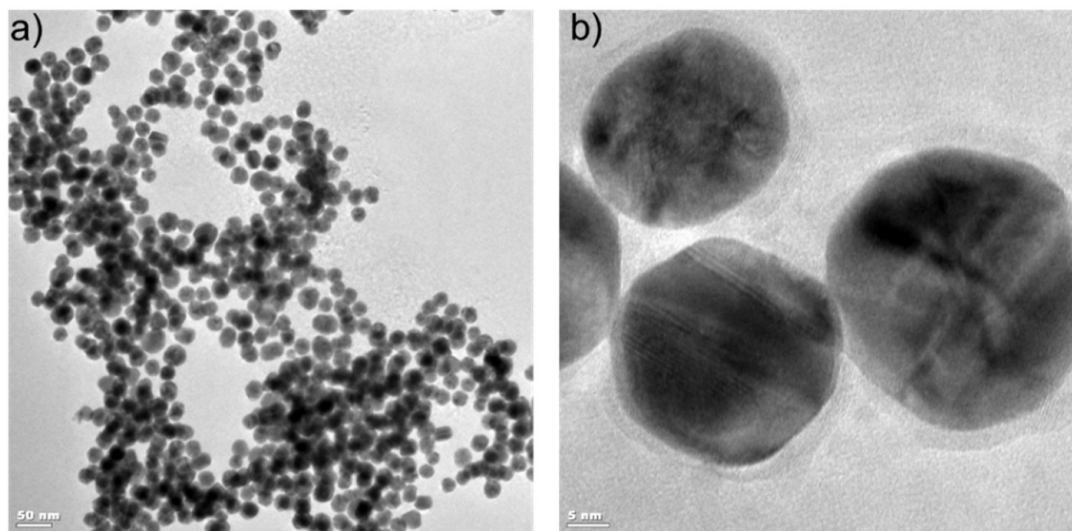


Figure 4.7: TEM image of gold nanoparticles.

4.1.4 Zetasizer Nano Zs

The Zetasizer Nano ZS (Malvern Panalytical, UK) utilizes the principle of Dynamic Light Scattering, DLS. This method calculates the Brownian motions

(Stokes–Einstein equation) by using the brightness and amounts of photons scattered from the particles in the sample and converts this information into particle diameter or radius [180].

Herein, the size of AuNPs was determined using a 488 nm laser. AuNPs were diluted with distilled water (1:10 dilution). The parameters for the measurement (refraction index of gold, absorption of gold, solvent type, temperature, and equilibrium time) were set as 0.2, 3.320, water, and 25 °C, 50 seconds, respectively. The diameter of AuNPs was observed as 35.59 nm (Figure 4.8a). As the DLS principle measures the hydration layer around the particles, we measured ~ 10 nm of a hydration layer during the measurements. To understand the seeding solution effect, 50 μL of 200 μM of seeding solutions (25 μL of 400 μM HAuCl_4 and 25 μL of 400 μM $\text{HONH}_2\cdot\text{HCl}$ with a 1:1 volume ratio) were added into 50 μL of stock AuNPs, and the remaining volume in the cuvette was filled with distilled water (until to reach 1 mL of total volume). The size of AuNPs was enlarged to 46.77 nm due to the effect of seeding solution (Figure 4.8b). Considering the hydration layer measurements for unmodified AuNPs and size measurements of unmodified AuNPs with TEM, we roughly calculated ~ 11 nm of enlargement in AuNP diameter after the seeding process.

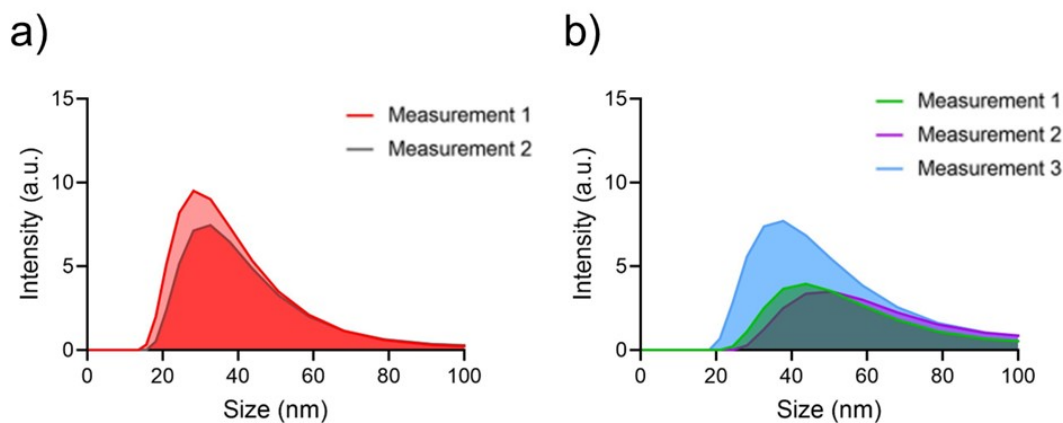


Figure 4.8: Zeta measurements for gold nanoparticles. (a) The plot represents the size (diameter) of AuNPs. (b) While applying the seeding solution into AuNP solution, the particle size becomes larger.

The NI was constructed on the PLL-modified surface without any particles; therefore, seeding solutions were mixed, and zeta measurements were performed to understand of the NI formation. At first, 50 μL of 400 μM of HAuCl_4 and 50 μL of 400 μM of $\text{HONH}_2\cdot\text{HCl}$ with a 1:1 volume ratio mixed and 900 μL of distilled water was added to complete the volume to 1 mL. Same measurement parameters were employed. The measurements started 10 minutes after the solutions were mixed, and the reaction continued during the measurement, The NI size was determined as 47.30 nm (Figure 4.9a). The same experiment was repeated with the 500 μM of HAuCl_4 and 500 μM of $\text{HONH}_2\cdot\text{HCl}$ with a 1:1 volume ratio (50 μL from each solution and 900 μL for distilled water) and the size of NI was determined as 49.34 nm (Figure 4.9b). The NI formation was defined as a time-dependent process as the formation and size of potential AuNPs increase proportional to the concentration of seeding solution.

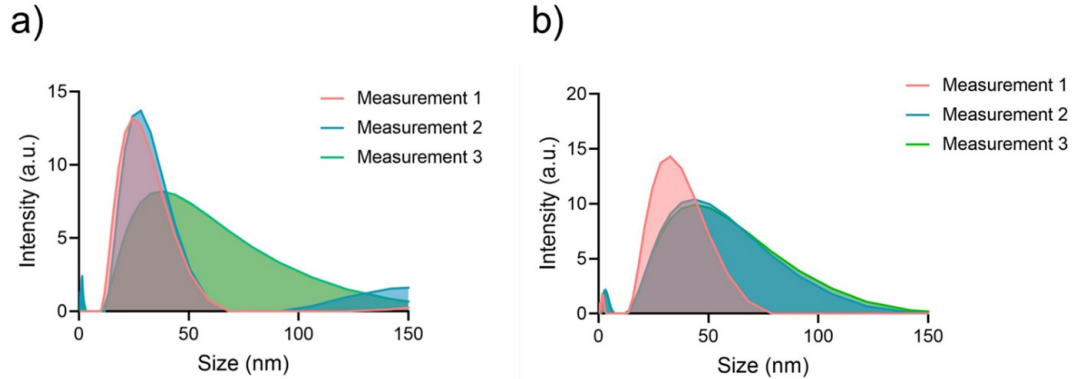


Figure 4.9: Zeta measurements for NI formation (a) 200 μM of seeding solution. (b) 500 μM of seeding solution.

Zeta potential reflects the colloidal stability of a particle in a suspension by measuring electrophoretic mobility [181]. This characterization method includes to measure the electrostatic potential at the electrical double layer surrounding a nanoparticle in solution [182]. The net charge of particles surface (determined by the zeta potential) provides information about particle – particle and particle – medium interactions and the tendency to aggregate particles [181]. Nonfunctionalized extracellular vesicles (including exosomes) have a negative net surface

charge due to expressed molecules at the surface [181]. In the literature, the exosome zeta potential was indicated as between -7 mV and -15 mV [183]. We also measured the zeta potential of the isolated exosome -6.06 ± 1.42 mV, matching with the values in the literature.

4.1.5 Nanoparticle Tracking Analysis: NanoSight NS300

Nano Tracking Analysis (NTA) (NS300, Malvern Instruments Ltd., Malvern, Worcestershire, UK) profiles the concentration, and size distributions of nano-sized particles such as exosomes. It utilizes a laser-based optical device that monitors the Brownian motion of single particle in a solution. In our setup, as suggested by the manufacturer, the embedded laser wavelength is 488 nm to image and to obtain the size information of exosomes [8].

To analyze exosome size and concentrations, the isolated exosomes were introduced to the sample chamber integrated with the CCD camera and 488 nm laser. The particles' diameter was quantified by solving the Stokes–Einstein equation. Before starting the measurement, distilled water was applied through the tubings to remove any possible impurities. For the analysis, viscosity (0.92 mPa.s), temperature (25 °C), and dilution rates were set before the measurements in the commercial software. Each measurement was executed with 5 videos for 60 seconds, and analyzed through the software.

The size distribution of exosomes was displayed in Figure 4.10, which states an average of 5 measurements (5 videos during 60 seconds of video recording). The mean size of exosomes was 85.2 ± 2.7 nm, and the mode was 71.1 ± 2.5 nm. The concentration was calculated as 4.30×10^8 particles/mL. Exosome size information was also matching their average size (87.25 ± 15.46 nm) calculated through the SEM (Figure 4.10). Stock solutions were diluted to 108 particles/mL with PBS for the detection experiments detailed in the next chapters.

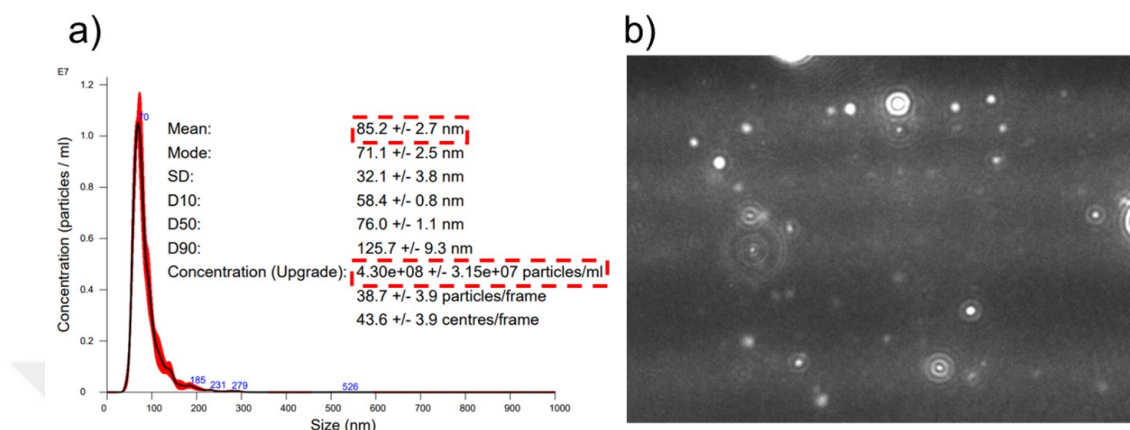


Figure 4.10: NTA analysis of the isolated particles. (a) Size distribution and concentration of the exosomes. (b) The image from the NTA recorded videos were used for measuring exosome size and quantifying their concentrations.

4.2 Chemical Characterization

The chemical characterization defines and determines the surface's elemental composition [184]. This study was mainly performed to confirm the success of surface modifications. Herein, contact angle measurements and XPS were employed for these purposes.

4.2.1 Contact Angle

The contact angle measurements are performed to determine hydrophilicity and hydrophobicity of a surface. The hydrophobicity is associated with surface energy. When the surface energy is low, the surface has hydrophobic properties. The contact angle is calculated by the angle between liquid or vapor and the solid surface interface. Small liquid droplets resting on a flat horizontal solid surface are basically used for the measurement. Young–Laplace equation determines the shape of the droplet. A contact angle goniometer is used for the measurements [179].

In this study, contact angle measurements were performed after the AuNPs-integrated and NI-formed surfaces. For these purposes, bare surface, PLL-modified surface, AuNP-integrated surface, and NI-formed surfaces were prepared. Measurements were carried out using Contact Angle Measurement System (Dataphysics, OCA 30, Filderstadt, Germany). In this process, 5 μL of ultrapure water was dropped on each surface, and the results were recorded. All measurements were performed three times, and their average and standard deviation were calculated.

In the gold top metasurface, the bare surface (unmodified surface) had a hydrophobic characteristic, and the contact angle was calculated as $99.63^\circ \pm 4.76^\circ$ (Figure 4.11a). After the PLL modification, the surface hydrophobicity decreased to a contact angle of $89.63^\circ \pm 1.1^\circ$ (Figure 4.11b). In contrast to PLL modification, integrating AuNPs or forming NI increased surface hydrophobicity to contact angles of $101.73^\circ \pm 1.86^\circ$ and $93.6^\circ \pm 1.3^\circ$, respectively (Figure 4.11c-d).

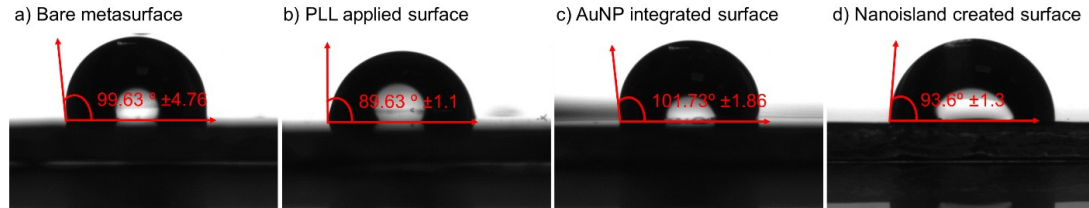


Figure 4.11: Contact angle analysis of (a) bare, (b)PLL applied, (c) AuNP integrated (d) nanol island created on gold top metasensor.

In the silver top metasurface, the bare surface had a hydrophobic characteristic along with a contact angle of $109^\circ \pm 2.5^\circ$ (Figure 4.12a). After the PLL modification, surface hydrophobicity was turned to hydrophilic characteristic with a contact angle of $62.01^\circ \pm 1.19^\circ$ (Figure 4.12b). Likewise, integrating AuNPs or forming NI increased surface hydrophobicity to contact angles of $66.93^\circ \pm 7.1^\circ$ and $86.37^\circ \pm 0.4^\circ$, respectively (Figure 4.12c-d) compared to the ones of the PLL modification.

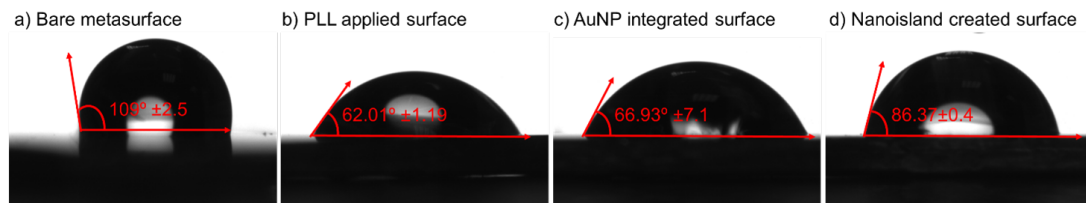


Figure 4.12: Contact angle analysis of (a) bare, (b) PLL applied, (c) AuNP integrated (d) nanoisland created on silver top metasensor.

As we applied same chemistry to both gold and silver top metasurfaces, we here only evaluated the contact angle values on the gold top metasurfaces. The gold top metasurface (bare surface) was sequentially functionalized with MUA, EDC/NHS chemistry for protein G, and anti-CD81 antibody immobilization. After modifying with MUA, surface hydrophobicity decreased due to carboxylic ends of this molecule ($54.7^\circ \pm 4.62^\circ$) (Figure 4.13a). The contact angle of the surface modified until protein G step ($78.06^\circ \pm 1.12^\circ$) also revealed more hydrophilicity (Figure 4.13b).

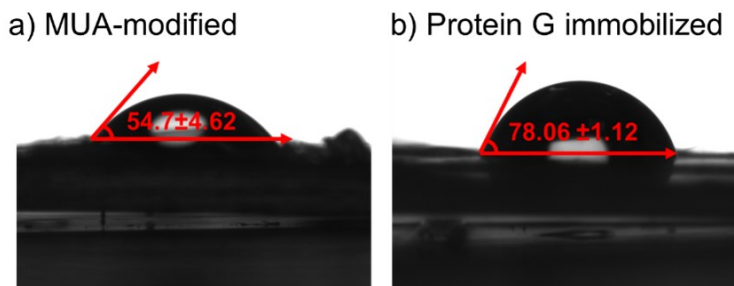


Figure 4.13: Contact angle of (a) MUA-modified, and (b) protein G-immobilized on gold top bare metasensor.

4.2.2 X-ray photoelectron spectroscopy (XPS) analysis

We performed XPS analysis (K-Alpha XPS, ThermoFisher Scientific, USA) to characterize the surface chemistry. XPS determines the material's elemental composition and chemical state through the photoelectric effect. Initially,

XPS analysis of Au4f and N1s scans were performed for the bare version of gold top metasurface (no surface chemistry applied) and then the modified versions (PLL modified surface, AuNP-integrated surface, and NI-formed surface). For the silver top metasurfaces addition to Au4f and N1s scans, Ag3d was also investigated. To understand the success of the layer-by-layer chemistry, the bare version of gold top metasurface, protein G immobilized surface (through MUA and EDC/NHS chemistry), anti-CD81 antibody-functionalized surface and exosome captured surfaces were scanned with Au4f, C1s and N1s.

For the gold top metasurface, N1s and Au4f scans were initially performed for the bare, PLL modified, AuNP-integrated, and NI-formed surfaces. The chemical structure of the PLL contains nitrogen; therefore, N1s scans were performed. N1s scan of the bare metasurface did not present any peak associated with nitrogen atoms (Figure 4.14a). When the surface is modified with the PLL, the N1s scan was associated with two bonds (imide and amide bonds) located at 401.95 and 400.35 eV due to the nitrogen content of the biopolymer (Figure 4.14b). After AuNP integration to the PLL-modified surface, we observed a reduction in the intensity of amide bond (at 399.4 eV) and also realized ~ 0.9 eV of a shift (Figure 4.14c). Similar to the AuNP integration, NI-formation on PLL-modified surface also reduced the intensity of amide bond (at 399.4 eV) along with a shift of ~ 0.9 eV compared to PLL-modified surface. When we examined the AuNP-integrated and NI-formed surfaces, there were no significant differences in terms of binding energies and intensity of the peaks (Figure 4.14d).

The Au4f scan of bare gold top metasurface provided two peaks accounting for Au ($4f_{5/2}$) and Au ($4f_{7/2}$) at the binding energies of 88.39 and 84.71 eV, respectively. The intensity was highest in comparison to the modified surfaces (Figure 4.14e). When the surface was modified with PLL, the intensities at Au ($4f_{5/2}$) and Au ($4f_{7/2}$) peaks (at 84.71 eV and 88.93 eV) reduced to the lowest level. In addition, they were shifted to 84.07 eV and 87.74 eV (Figure 4.14f). After the AuNP integration, the intensity of the Au ($4f_{5/2}$) and Au ($4f_{7/2}$) peaks at 87.19 eV and 83.51 eV elevated compared to PLL-modified surface, and the peaks shifted ~ 0.5 eV. In the case of NI-formed surface, Au ($4f_{5/2}$) and Au ($4f_{7/2}$) intensities

were similar to the AuNP-integrated surface, but they were higher than the ones in PLL-modified surface. Moreover, the peaks were shifted ~ 0.5 eV compared to AuNP-integrated surface.

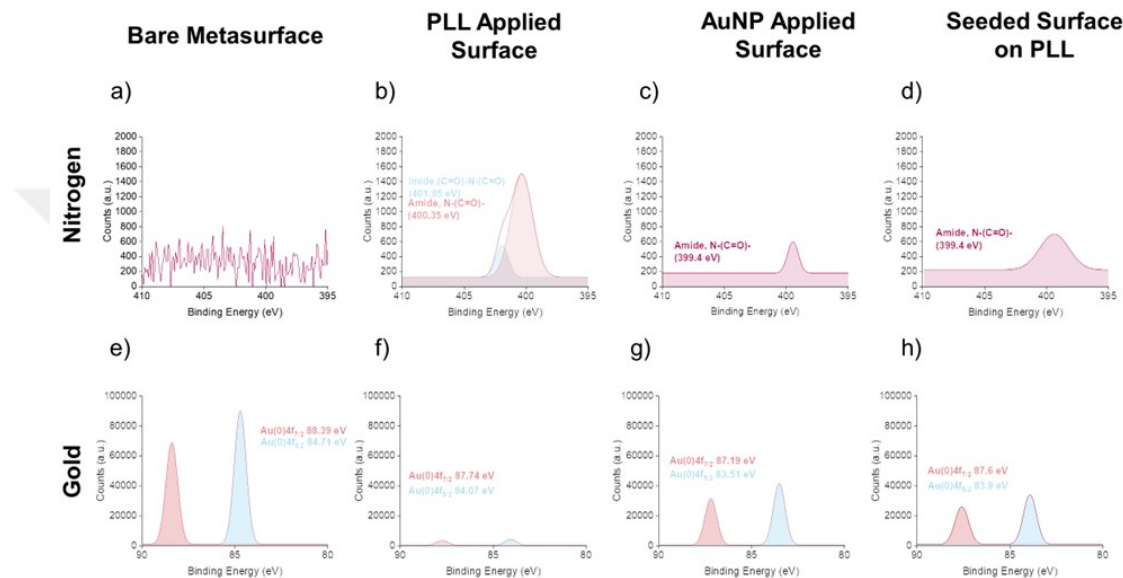


Figure 4.14: XPS characterization for the surfaces. N1s scans are presented for (a) bare gold top metasurface, (b) PLL-modified surface, (c) AuNP-integrated surface, and (d) NI-formed surfaces. Au4f scans are shown for (e) bare gold top metasurface, (f) PLL-modified surface, (g) AuNP-integrated surface, and (h) NI-formed surfaces.

Considering the silver top metasurface, N1s, Ag3d and Au4f scans were performed for the bare, PLL-modified, AuNP-integrated, and NI-formed surfaces. Similar to the bare gold metasurface, the bare silver top metasurface did not exploit any nitrogen-associated peaks (Figure 4.15a). The PLL-modified surface provided two peaks associated with the imide and amide bonds at 400.19 eV and 399.01 eV, respectively (Figure 4.15b). The modifications shifted the binding energies to 400.02 eV and 401.80 eV for the integration of AuNPs, as well as 399.83 eV and 401.6 eV for the formation of NI, respectively (Figure 4.15c-d).

Again, for the silver top metasurface, the scan of Ag3d was employed for the bare

metasurface, PLL-modified surface, AuNP-integrated surface, and NI-formed surface. Regarding the Ag3d scan, the silver surface presented two peaks associated with Ag ($3d_{3/2}$) and Ag ($4d_{5/2}$) at the binding energies of 368.21 eV and 374.22 eV, respectively (Figure 4.15e). PLL modified surface provided Ag ($3d_{3/2}$) and Ag ($4d_{5/2}$) peaks at 368.07 eV and 374.07 eV binding energies. AuNP-integrated and NI-integrated surfaces also presented Ag ($3d_{3/2}$) and Ag ($4d_{5/2}$) at the binding energies of 368.27 eV and 374.27 eV; 368.27 eV and 374.28 eV, respectively. Regarding the Au4f scan, the bare silver top and PLL-modified metasurfaces did not provide any peak associated with the Au ($4f_{5/2}$) and Au ($4f_{7/2}$) as expected. After integrating AuNP and forming NI, we started to observe Au ($4f_{5/2}$) and Au ($4f_{7/2}$) peaks at the binding energies of 87.81 eV and 83.16 eV; 87.50 eV and 83.84 eV (Figure 4.15k-l).

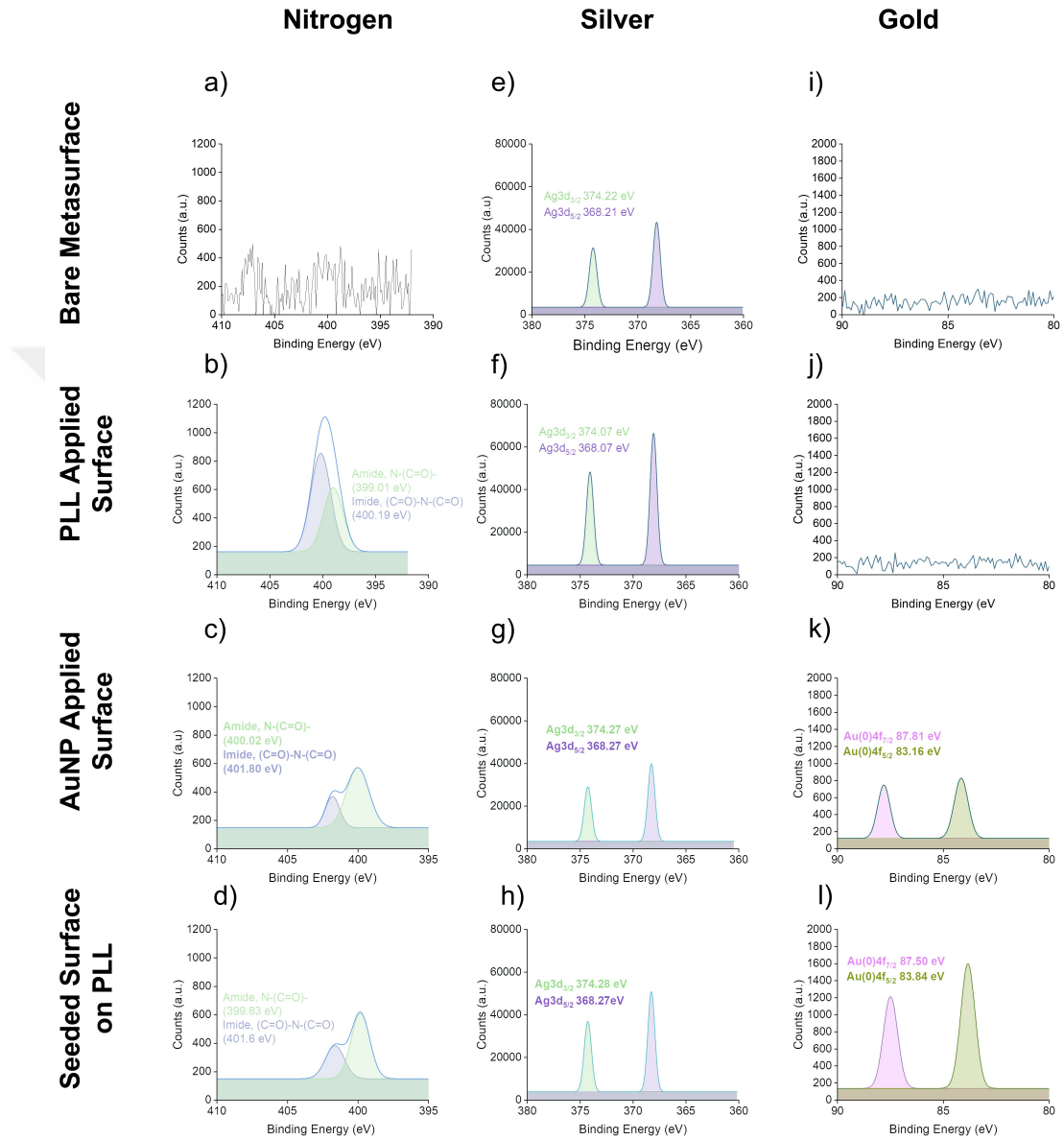


Figure 4.15: XPS characterization for the surfaces. N1s scans are presented for (a) bare silver top metasurface, (b) PLL-modified surface, (c) AuNP-integrated surface, and (d) NI-formed surfaces. Ag3d scans are shown for (e) bare silver top metasurface, (f) PLL-modified surface, (g) AuNP-integrated surface, and (h) NI-formed surfaces. Au4f scans are presented for (i) bare silver top metasurface, (j) PLL-modified surface, (k) AuNP-integrated surface, and (l) NI-formed surfaces.

Since we run the same surface chemistry to both gold top and silver top metasurfaces, we only performed XPS analysis of C1s, Au4f, and N1s scans for the bare sensor (no surface chemistry applied) and protein G, anti-CD81 antibody immobilized surface and exosome captured surface on the bare gold top metasurface. The Au4f scans of bare gold top metasurface presented two peaks associated with Au ($4f_{5/2}$) and Au ($4f_{7/2}$) at the binding energies of 84.32 ± 0.005 eV and 88.0 ± 0.005 eV, respectively. When protein G was immobilized to the surface through (i) MUA and (ii) EDC/NHS chemistry, Au ($4f_{7/2}$) and Au ($4f_{5/2}$) peaks at 83.97 ± 0.004 eV and 87.65 ± 0.005 eV of binding energies slightly shifted (~ 0.4 and ~ 0.6 eV). The intensity of the peaks also reduced due to the immobilization of the protein G, pointing out the successful immobilization (Figure 4.16b). C1s scan indicated three peaks associated with C-C/C-H, C-O, and C=O that are major chemical groups in the molecular structure of protein G, anti-CD81 antibodies, and exosomes. The bare sensor provided two peaks, (i) C-C/C-H at 284.76 ± 0.01 eV and (ii) C=O at 287.60 ± 0.13 eV, which were shallow peaks with lower intensities (Figure 4.16e) compared to the ones in protein G data (Figure 4.16f). Protein G-immobilized surface presented three peaks: C-C/C-H at 284.77 ± 0.006 , C-O at 285.27 ± 0.05 eV, and C=O at 285.27 ± 0.05 eV (Figure 4.16f). The bare gold top metasurface did not indicate any peaks associated with the N1s scan (Figure 4.16i). When protein G was immobilized, a peak was observed at 400.30 ± 0.12 eV due to peptide bonds ($-\text{NH}-\text{C}(=\text{O})-$) (Figure 4.16j).

Regarding the antibody step, the Au4f scan provided two peaks associated with Au ($4f_{7/2}$) and Au ($4f_{5/2}$), located at the binding energies of 87.83 ± 0.004 eV and 84.15 ± 0.003 eV, respectively (Figure 4.16c). Once the exosomes were captured on the sensors decorated with anti-CD81 antibody, the Au ($4f_{7/2}$) and Au ($4f_{5/2}$) binding energies were 87.82 ± 0.005 eV and 84.15 ± 0.003 eV, respectively (Figure 4.16d). No significant differences were observed in the binding energies of Au for the sensor with the anti-CD81 antibody. The peaks maintained their positions along with lower intensities (counts) when the exosomes were captured. Similarly, in the C1s XPS scan, we observed similar peak locations (iii) C-C/C-H at 284.78 ± 0.006 , C-O at 285.63 ± 0.08 , and C=O at 288.76 ± 0.05 for anti-CD81 antibody-decorated sensor (Figure 4.16g), and (iv) C-C/C-H at 284.83 ± 0.006 ,

and C-O 286.39 ± 0.03 and C=O at 288.34 ± 0.07 once exosomes were captured on the sensor through anti-CD81 antibodies (Figure 4.16h). On the other hand, there were some shifts in the binding energy related to C=O (~ 0.68 and ~ 0.42 eV) after the exosomes were captured.

While the N1s results in the anti-CD81 antibody plot were examined, only C-N bond appeared at 399.76 ± 0.042 eV energy level was visible (Figure 4.16k). Considering the results of the exosome captured surface through anti-CD81 antibody, there were also C-N, N-H, and N-O bonds at 400.11 ± 0.13 eV, 400.93 ± 7.71 eV, and 399.38 ± 0.07 eV, respectively (Figure 4.16l). The outcomes from two distinct, but complementary analyses confirmed the successful construction of our surface chemistry on the sensor surface, and we continued the experiments accordingly.

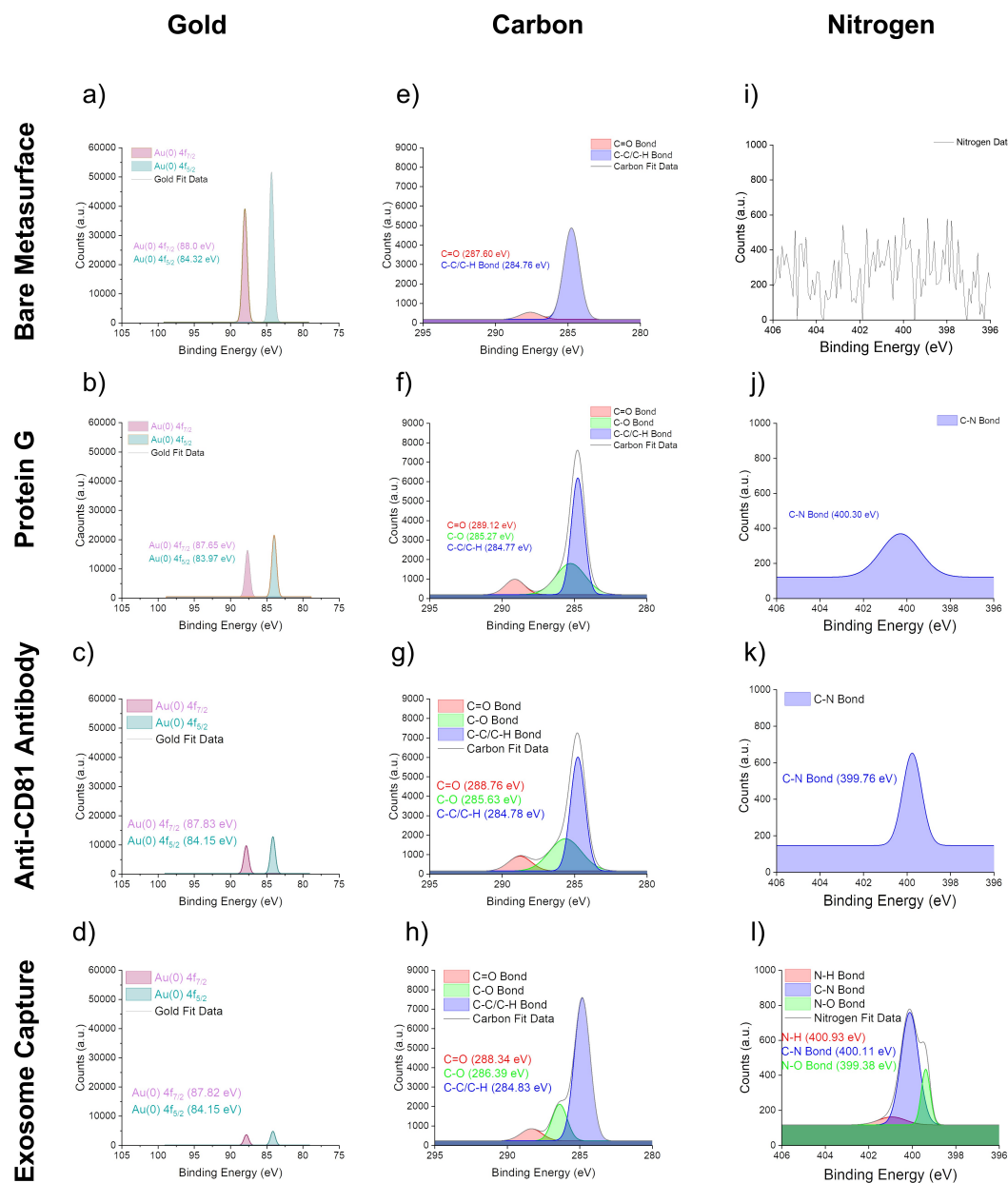


Figure 4.16: XPS characterization for the surface chemistry and exosome detection. Au4f scans are presented for (a) bare gold top metasurface, (b) protein G-immobilized surface, (c) anti-CD81 antibody immobilized surface, and (d) exosome captured surfaces. C1s scans are shown for (e) bare gold top metasurface, (f) protein G-immobilized surface, (g) anti-CD81 antibody immobilized surface, and (h) exosome captured surfaces. N1s scans are presented for (i) bare gold top metasurface, (j) protein G-immobilized surface, (k) anti-CD81 antibody immobilized surface, and (l) exosome captured surfaces.

4.3 Exosome Protein Characterization

4.3.1 Western Blot Analysis

Tetraspanins (CD63, CD81 and CD9) locate at the membrane of exosomes, and they are used as markers for exosome detection (Fang vd., 2013; Gang vd., 2021). Once exosomes are isolated from the microfluidic chip, we then validated them through western blot analysis by focusing their surface markers (CD63 and CD81). Here, the isolated exosomes were prepared in three different samples. Sample-1 was prepared after lysing exosomes with RIPA buffer that contains protease inhibitor. This step was followed by a centrifugation at 14,000 g for 15 minutes (+4 °C) and the supernatant was used for the western blot analysis. For preparing Sample 2, exosome lysate was not centrifuged, and all the lysate content was used for the analysis. Lastly, Sample 3 was defined as intact exosomes without any lysis and centrifugation processes. As a positive control, cell lysate was used by following the protocol of Sample 1.

In the literature, CD63 is mostly observed as a smear locating between 30 kDa and 60 kDa due to high glycosylation [185], whereas the corresponding band of CD81 is at 20-28 kDa [185, 186]. Per our results, we observed a smear band for CD63 protein within the range of 30-60 kDa (Figure 4.17a), and CD81 was visible at ~25 kDa (Figure 4.17b). Accordingly, we demonstrated success isolation of exosomes for further experiments.

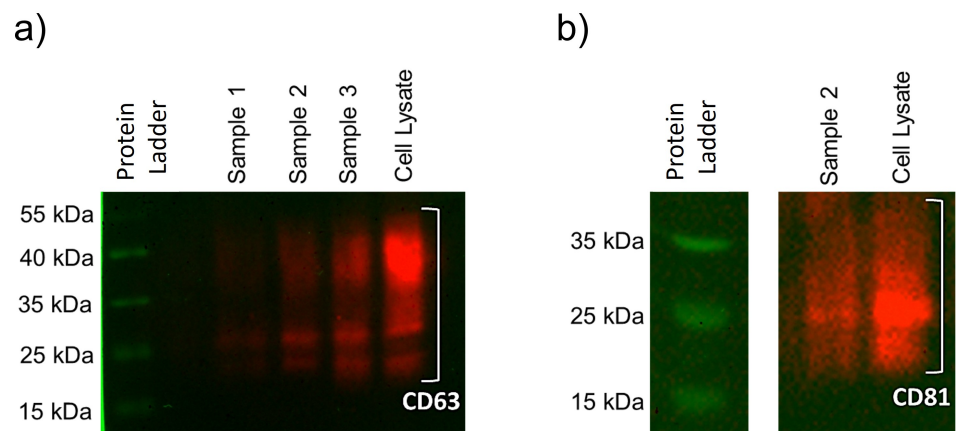


Figure 4.17: Exosome characterization with Western blot analysis. (a) CD63 protein and (b) CD81 protein were analyzed and shown in the analysis.

Chapter 5

Bulk Sensitivity Measurements

5.1 Portable Setup

After developing the optical setup and fabricating the sensors, we then benchmarked their performances. Initially, various glycerol solutions ranging from 1% to 70% were applied to the sensors, including the bare versions of silver and gold top surfaces, AuNP-integrated versions of silver and gold top surfaces, NI-formed versions of silver and gold top surfaces. Benchmarking of the setup also included while processing the data. The signal was obtained from the reflected light from the sensor surface. First, the data processing started with the recording of a blank channel (filled with air) and subtracted with the signal that was obtained from the sample-applied channel (Figure 5.1a). The blue graph belongs to the sensor+DSA+air+PMMA layer, the gray line is obtained from the sensor+DSA+sample (water in this case)+PMMA layers. The red line in the Figure 5.1a is achieved by subtracting background data from the incoming data. The peak of the red line is the resonance of the sensor that is followed for the wavelength shift. The second data processing strategy records the mirror as a background ($\sim 100\%$ of reflectance) and dividing the sample signal (Figure 5.1b).

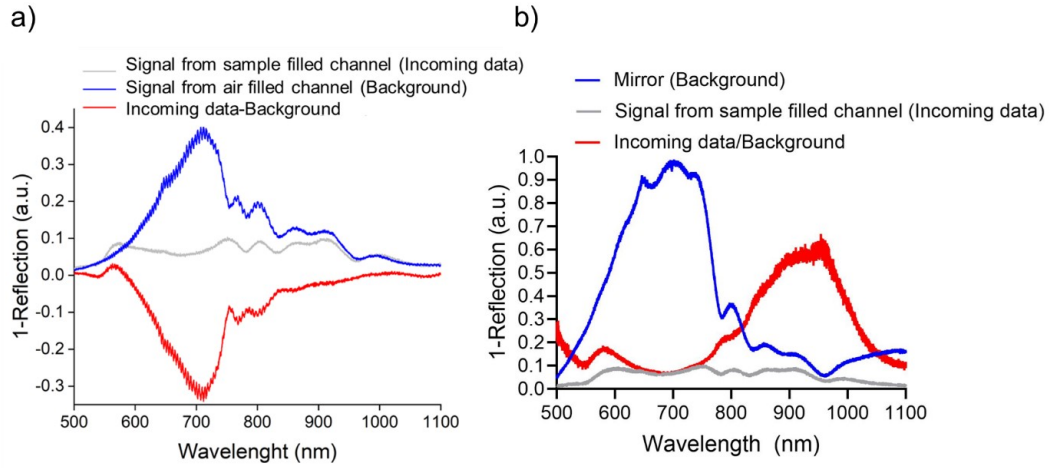


Figure 5.1: Types of data processing. (a) Subtracting and (b) dividing strategies are presented.

5.2 Bulk Sensitivity

While exploring the performance of the plasmonic sensor platforms, two concepts have been considered: (1) bulk sensitivity and (2) surface sensitivity. Although both concepts are associated with plasmonic electric-field distributions, they are also fundamentally different [187]. The bulk sensitivity can be described as the measurement of refractive index changes in spatially infinite (within accessible volume of the plasmonic electric field) [187]. Sensor performance was numerically indicated by the resonance full width at half-maximum (FWHM) and figure of merit (FoM). Moreover, bulk sensitivity does not reflect sensor sensitivity by itself, and the sensitivity is also dependent on the size of the captured molecules and the target analytes [187].

In this chapter, the bulk sensitivity of the fabricated sensors was evaluated with a gradient of the glycerol solutions, which altered RI values at the close vicinity of the sensor surface without any molecule binding. The first strategy was to fabricate nanograting-based sensors by coating the optical disks with Ti, Ag, and Au by thermal evaporation, termed gold top metasurfaces. Then,

AuNPs were integrated to this metasurface through a biomaterial layer (PLL). Followingly, NI was created on the nanograting with a similar strategy to AuNPs integration. We here also optimized the concentrations of PLL, AuNPs, and NI (seeding) solutions. After determining the optimum concentrations, the same procedure was applied to silver-coated sensors, termed silver top metasurfaces.

5.2.1 Sensor Performance of Bare Surfaces

As explained in previous chapters, two different nanograting-based sensors were fabricated by recycling optical disks. The first surface has a gold top, and the second surface has a silver top. After the surface fabrication, they were cleaned with ethanol (sonication 5 minutes), assembled with microfluidics, and prepared for the optical measurement. As explained above, two data processing strategies were employed for the bare gold top surface and continued to one approach based on the results. In the first strategy, the background was obtained from the empty channel of the sensor, then it was subtracted from the measurement second channel (sampling). To assess bulk sensitivity, glycerol solution was applied to the channels, which created RI changes on the surface. The stock glycerol was diluted with distilled water with different ratios starting from 1% to 70%. The baseline was created with the water for 200 seconds at 10 $\mu\text{L}/\text{minutes}$ of flow rate. When the baseline was obtained, each glycerol solution was introduced in an order for 200 seconds at 10 $\mu\text{L}/\text{minutes}$ of flow rate. Both the blank and sample-applied channels were measured on a portable spectrometer (THORLABS, CCS175/M,) by scanning the wavelength within the range of 500-1100 nm. The fabricated sensor indicated two resonances in this region: (i) a dip at 528.83 nm and (ii) a peak at 569.12 nm for the water. The noise of the resonances was eliminated with the smooth function in the analysis window of OriginPro by using the adjacent-averaging function by 100 points of window parameter. While applying the glycerol solutions (1-70%), these resonances were shifted (Figure 5.2a). In Figure 5.2b, the heatmap plot represented plasmonic signals proportional to glycerol concentrations (the black arrow indicated the

peaks shift). These measurements were also performed in real-time by our in-house software (Figure 5.2c). Once the real-time measurements were performed, wavelength shifts were calculated by taking an average of 100 seconds. Then, the shifts were plotted based on the RI changes, and linear correlations were obtained (Figure 5.2d). Refractive index sensitivity (S) is another parameter to evaluate the sensitivity of the sensors, which is calculated by Equation 1 [188]. For the bare gold top sensor with the subtracting data processing, the S value was calculated as 388.456 nm/RIU.

$$\text{RefractiveIndexSensitivity} = \frac{\Delta\lambda}{\Delta n} \quad (5.1)$$

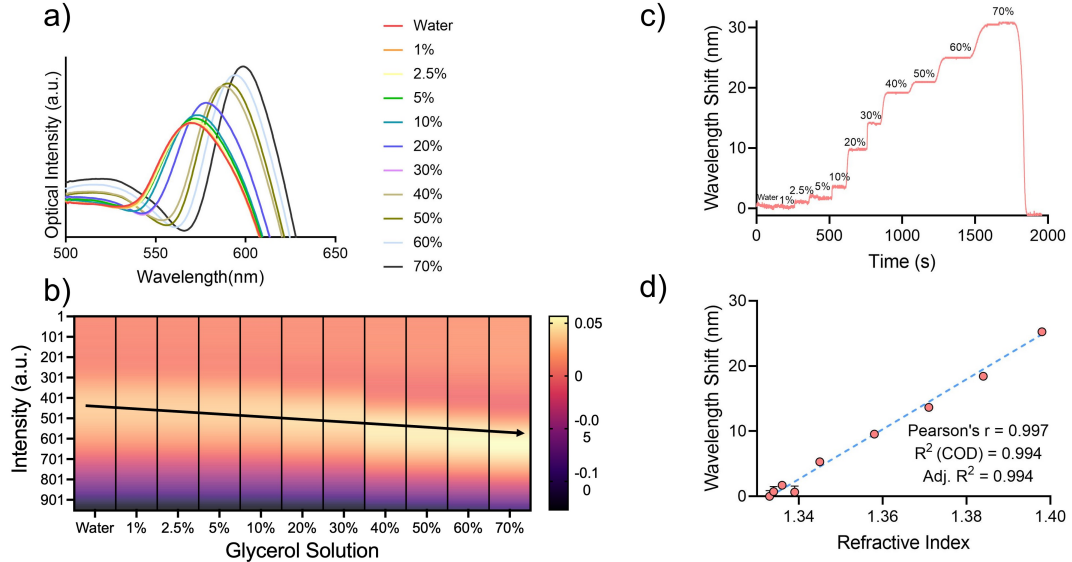


Figure 5.2: Benchmarking the plasmonic sensor performance of bare gold top metasurface. Subtracting strategy is performed for the data processing. (a) Changes in plasmonic resonance while applying different concentrations of glycerol solutions (1%-70%) and collecting the data as end-measurement. (b) A heatmap representation of the changes in plasmonic resonance while applying different concentrations of glycerol solutions (1%-70%). (c) Real-time measurements of the changes in plasmonic resonance while applying different concentrations of glycerol solutions (1%-70%) (d) A linear correlation plot for representing the changes in plasmonic resonance proportional to the refractive index values.

In the second strategy, the background was obtained from the mirror, and measurement was performed in the channel. Besides background and data processing, all experimental setups followed previous protocols. Herewith, the bulk sensitivity was calculated by applying 1-70% glycerol solutions and wavelength shifts. The baseline was created with the water for 200 seconds at 10 μ l/minutes with a syringe pump. When the baseline was obtained, each glycerol solution was introduced in sequence for 200 seconds at 10 μ l/minutes. Sample applied channels were measured on a portable spectrometer (THORLABS, CCS175/M,) by scanning the wavelength within the range of 500-1100 nm. The fabricated sensor indicated two resonances in this region: (i) a dip at 531.25 nm and (ii) a peak at 554.47 nm for the water (Figure 5.3a). The noise of the spectrum was eliminated by averaging 100 points in OriginPro software. Heatmap representation, real-time measurement, and linear relationships were plotted the same way as the first strategy (Figure 5.3). The S value was calculated as 405. 47 nm/RIU. Considering the S value, further experiments were conducted using the mirror as a background.

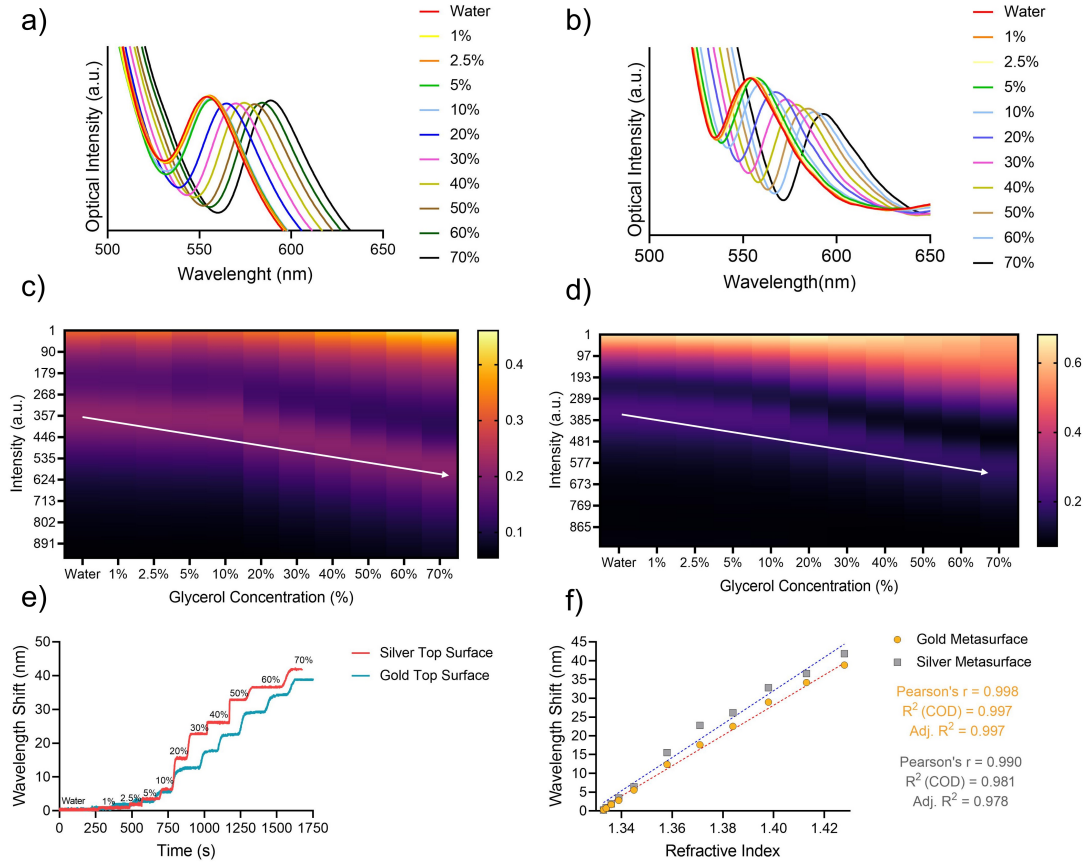


Figure 5.3: Benchmarking the plasmonic sensor performance of bare gold top and bare silver top metasurface. Dividing strategy is performed for the data processing. (a) Changes in plasmonic resonance while applying different concentrations of glycerol solutions (1%-70%) and collecting the data as end-measurement for the gold top metasurface. (b) Changes in plasmonic resonance while applying different concentrations of glycerol solutions and collecting the data as end-measurement for the silver top metasurface. (c) A heat-map exhibits the changes in plasmonic resonance of the gold top metasurface while applying different concentrations of glycerol solutions on gold top metasurface. (d) A heat-map represents the changes in plasmonic resonance of the gold top metasurface while applying different concentrations of glycerol solutions on silver top metasurface. (e) Real-time analysis demonstrates the changes in plasmonic resonance value of different glycerol solutions on gold top metasurface and silver top metasurface. (f) Linear correlation plot represents of the changes in plasmonic resonance value based on refractive index changes for gold top metasurface and silver top metasurface.

After determining the data processing, the bulk sensitivity of the fabricated silver top metasurface was performed with the second strategy. The baseline was created with water and 1-70% glycerol solutions were applied to the channel. The spectrum was scanned between 500-1100 nm. The fabricated sensor indicated two resonances in this region: (i) a dip at 534.75 nm and (ii) a peak at 554.17 nm for the water (Figure 5.3a). The noise of the spectrum was eliminated by averaging 100 points in OriginPro software. Heatmap representation, real-time measurement, and linear relationships were plotted the same way as the first strategy (Figure 5.3). The S value was calculated as 438.07 nm/RIU. Considering the S value of the empty surfaces, the silver top surface indicates higher sensitivity in comparison to the gold top surface.

Up to now, different strategies have been applied to enhance the sensor performance in plasmonic fields, such as nanoparticle combination, nanostructure modification, etc. In this study, two different approaches were utilized to enhance sensitivity. The first approach was to integrate AuNPs onto the nanograting structure, and the latter was to form NI through the seeding reactions on PLL-integrated sensor. In the beginning, the creation of the NI was tried on integrated AuNPs by enlarging with seeding solutions; however, this approach did not work well. Afterward, seeding solutions were directly applied to the PLL-coated surface. The main challenge of forming these modifications on the nanograting was damaging the nano periodic structure (width and spaces). While determining the optimum parameters for the AuNPs and NI, bulk sensitivity was taken into account by applying glycerol solutions.

5.2.2 Gold Nanoparticle Integrated Metasurfaces

To enhance the plasmonic signal of the fabricated metasurface-based sensors, synthesized AuNPs (~ 30 nm diameter) were integrated onto the nanograting structures through the PLL layer. Fabricated sensors were cut into 15×15 cm² with scissors and cleaned with ethanol. The surface was modified by incubating in 0.5 mg/mL of PLL solution at +4 °C overnight. PLL-modified surfaces were

incubated in different diluted AuNPs stock solutions (1:2, 1:5, 1:10, 1:20, and 1:50 dilution ratio) at +4 °C overnight. A high concentration of the gold nanoparticle solutions reduced the resonance signal due to the deterioration of the nanoporous structure. In Figure 5.4, the surface was incubated with the 1:2 diluted stock of AuNP solution, and the resonance was lost.

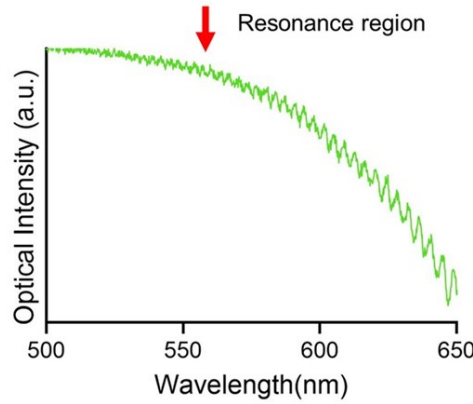


Figure 5.4: The plasmonic resonance of a sensor is lost due to high AuNPs integration.

When the sensor surface was incubated in 1:5 diluted stock of AuNP solution, the resonance was obtained. Therefore, 1-30% glycerol solution was applied to the surface to evaluate bulk sensitivity after creating a baseline with distilled water. Followingly, 1:10, 1:20, and 1:50 dilutions were performed using the same experimental protocol. To compare their sensitivity, linear correlations were demonstrated in Figure 5.5 by plotting RI change versus wavelength shift. The results indicated that a 1:10 dilution of the stock AuNP solution led to higher signal enhancement compared to the counterparts. Briefly, the S values were 391.76 nm/RIU for 1:5 dilution ratio, 496.84 nm/RIU for 1:10 dilution ratio, 353.16 nm/RIU for 1:20 dilution ratio, 365.84 nm/RIU for 1:50 dilution ratio. When 1:10 dilution AuNP was integrated, the S value was enhanced ~ 1.3 times compared to the bare version.

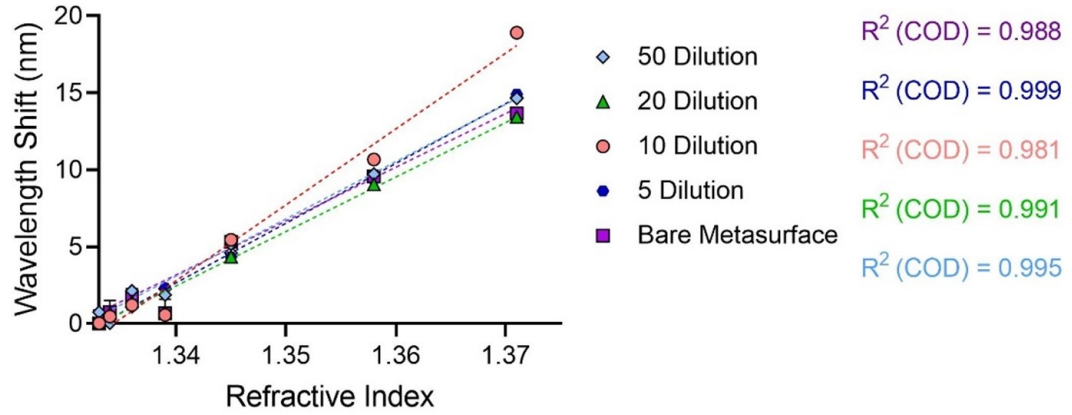


Figure 5.5: Linear correlation of gold nanoparticle amount on the plasmonic resonance while changing the refractive index of bulk condition through various concentrations of glycerol on gold top metasurface.

After optimizing the AuNPs amount and PLL concentration, the silver top metasurface was also fabricated with the same protocol. The S value of the silver top metasurface increased from 438.07 nm/RIU to 515.75 nm/RIU, resulting in ~ 1.18 of signal enhancement compared to the bare version. When the both AuNPs-integrated surfaces were compared silver top metasurface indicated higher S value which means higher bulk sensitivity whereas the gold top metasurface resulted in more enhancements (~ 1.3) than its bare version (Figure 5.6).

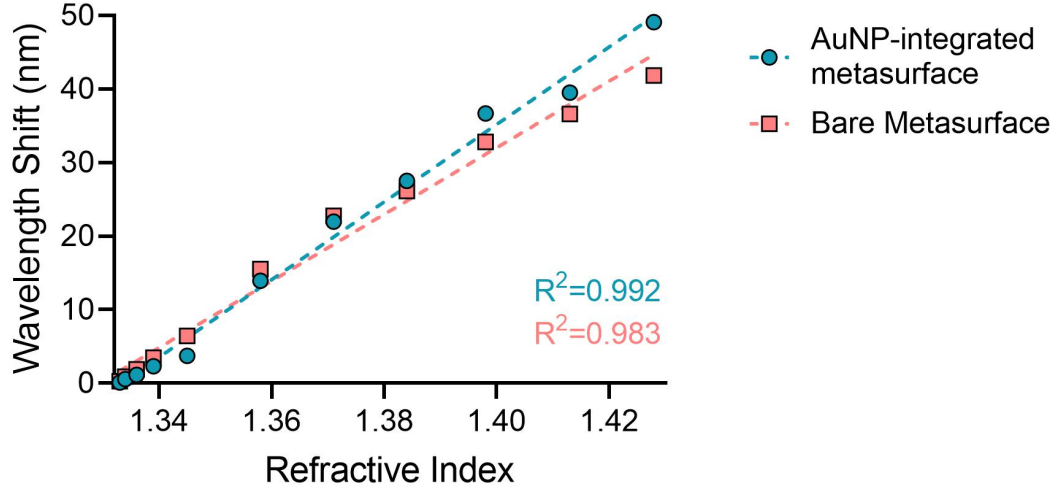


Figure 5.6: Comparison of the bare silver top and AuNPs-integrated silver top metasurface.

5.2.3 Nanoisland-Formed Surfaces

In the second enhancement strategy, NI was formed on the nanoperiodic structure with a chemical reaction. The construction of the NI was prepared by the reduction of chloroauric acid (HAuCl_4) with hydroxylamine hydrochloric acid ($\text{HONH}_2\cdot\text{HCl}$) [89]. The procedure enables to form AuNPs and seed them at the same time by the hydroxylamine reduction of HAuCl_4 on the PLL-modified gold nanostructure. The same process was applied to the uncoated surface; yet any signal changes were observed. In addition, the NI formation was also attempted on the AuNP-integrated surface. When the seeding solution chloroauric acid (HAuCl_4) and hydroxylamine hydrochloride ($\text{HONH}_2\cdot\text{HCl}$) were applied, any significant signal changes were however observed (Figure 5.7).

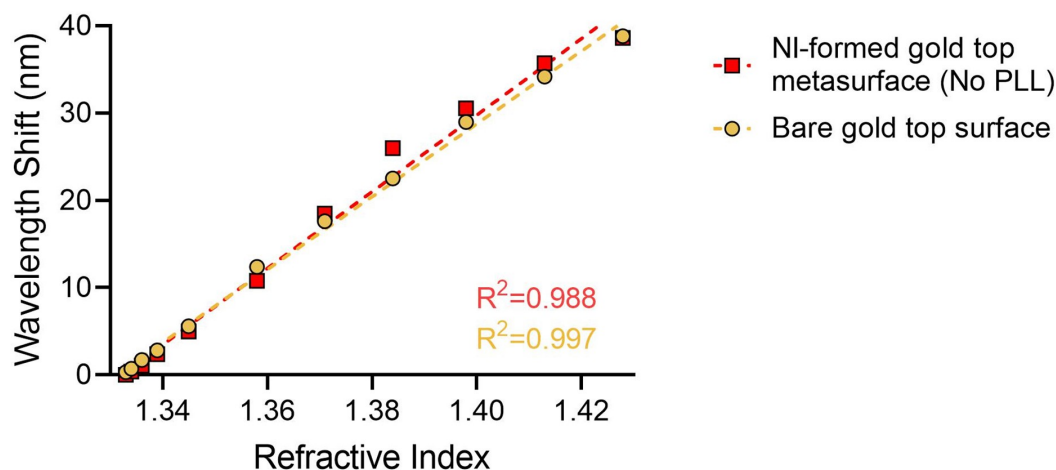


Figure 5.7: Wavelength shifts proportional to the changes in refractive index in the presence and absence of PLL-modification while forming NI on the bare gold surface.

The main crucial part of the NI formation is to preserve nanoperiodic structure (width or spaces). Applying excessive concentration of the seeding solution or a long incubation would possibly damage the nanoperiodic design, leading to signal loss. Herein, 0.5 mg/mL of PLL modified gold top surface was combined with the microfluidic chip and optical measurements were obtained. As demonstrated in Figure 5.8, 100 μ M seeding solution (200 μ M of HAuCl_4 and 200 μ M of $\text{HONH}_2\cdot\text{HCl}$) was applied to the channel for 5 minutes after creating a baseline with distilled water. When the seeding solution entered the surface, NI formation shifted wavelength since the structure of the nano periodicity was rearranged. As indicated in Figure 5.8a, when the seeding solution flowed ~ 2 minutes at 10 $\mu\text{L}/\text{minutes}$, the wavelength reached the maximum (shifted ~ 10 nm). After 2 minutes wavelength was back shifted. As shown in Figure 5.8b, the resonance intensity also decreased, and after 5 minutes, it was lost; hence, the optimization of the solution concentration and duration provided a more controllable construction of the NI.

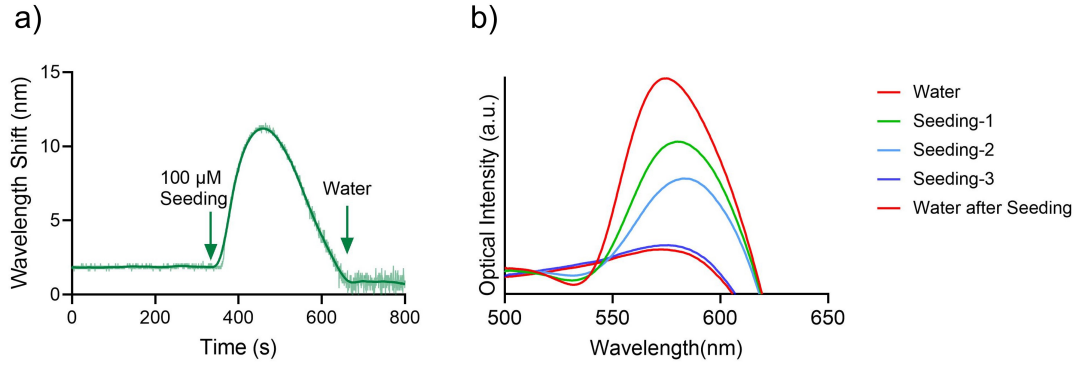


Figure 5.8: The effect of seeding solution on PLL (0.5 mg/mL)-modified gold top surface. (a) Real-time measurement of the NI formation. (b) Resonance changes while applying seeding solution.

To understand the limits of our signal enhancement strategy, various concentrations of the seeding solutions were applied to 0.5 mg/mL of PLL-modified gold top metasurface. Therefore, 10 μM , 20 μM , 50 μM , and 100 μM seeding solutions were applied for 5 minutes and washed to stop the reaction. In Figure 5.9, 10 μM of seeding (mixing 20 μM of HAuCl_4 and 20 μM of $\text{HONH}_2\cdot\text{HCl}$ with a 1:1 (v:v) ratio) provided a higher S value. For instance, the S value of the 100 μM of seeding was 421.74 nm/RIU; 50 μM of seeding resulted in 433.05 nm/RIU; 20 μM of seeding caused to 525.87 nm/RIU; and 10 μM of seeding led to 546.42 nm/RIU. When we reduced the concentration less than 10 μM , no significant enhancements were attained. When the incubation time for 10 μM of seeding was increased, the S value also started to decline. Considering these results, 10 μM seeding along with 5 minutes of incubation was the highest SI value while working with NI-formation on the gold top metasurface. The S value was enhanced ~ 1.35 times compared to the bare metasurface.

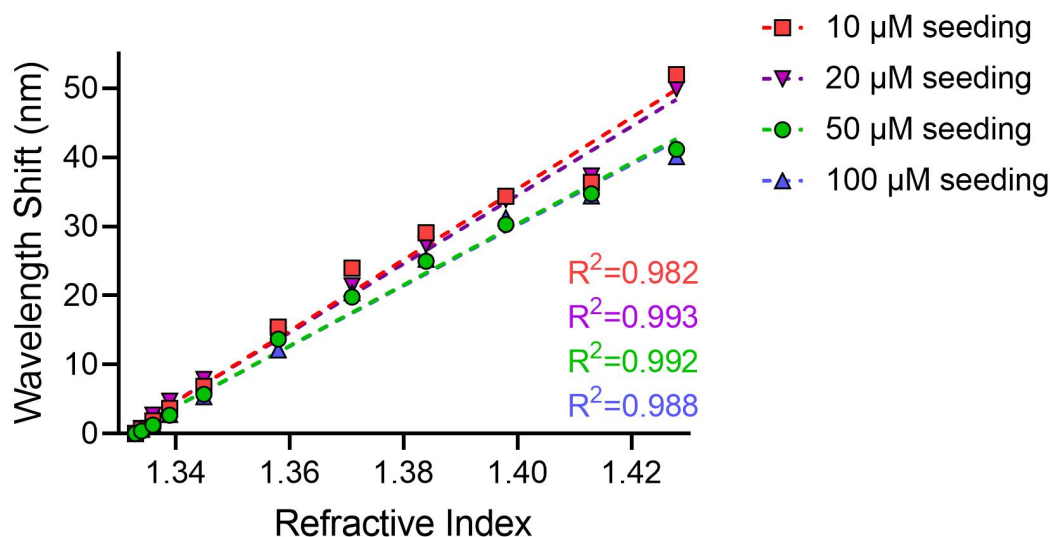


Figure 5.9: Evaluating the effect of seeding solution concentration on PLL (0.5 mg/mL)- modified surface for the NI formation and benchmarking the analytical performance while changing the bulk refractive index.

As the control experiment pointed out, without PLL modification, there was no NI formation and signal enhancement. Considering all these facts, the concentration of the PLL is also another parameter to tune this strategy; hence the optimized concentration of seeding solution was tried on varying PLL concentrations (1, 0.5, 0.1, and 0.05 mg/mL). When we compared the S value of each condition, the highest S value was achieved while utilizing 0.5 mg/mL of PLL along with 10 μ M of seeding (546.42 nm/RIU), and the lowest S value was obtained from 1 mg/mL of PLL-modified surface (500.96 nm/RIU) (Figure 5.10).

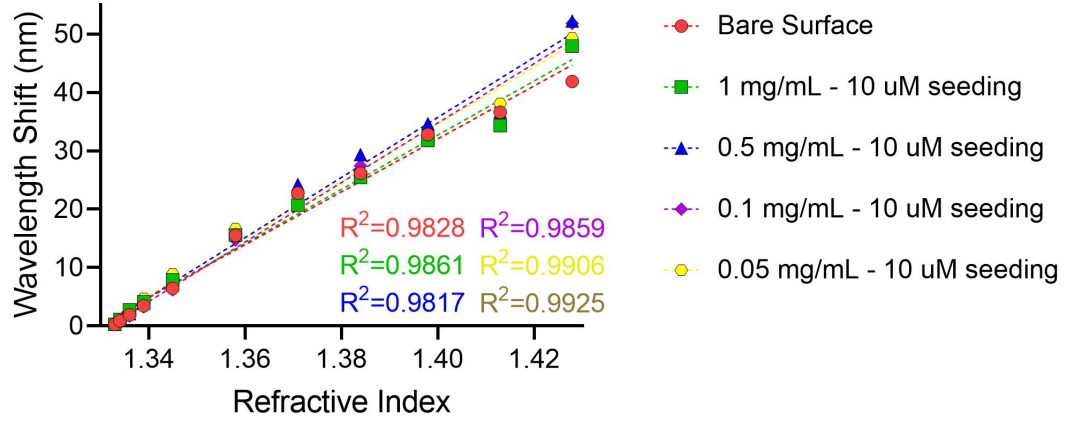


Figure 5.10: Evaluating the effect of PLL concentration for the NI formation and benchmarking the analytical performance while changing the bulk refractive index.

The NI-formation parameters were set with the gold top metasurfaces as 0.5 mg/mL of PLL modification and 10 μ M of seeding solution for 5 minutes. Once all parameters were optimized, they were applied to the silver top metasurfaces as well. Silver top metasurfaces were modified with 0.5 mg/mL of PLL, and the NI formation was performed with a 10 μ M of seeding solution (20 μ M of HAuCl_4 and 20 μ M of $\text{HONH}_2\cdot\text{HCl}$ 1:1 v:v ratio). The NI-formed surface had 1.12 times lower S value than that of the bare silver top metasurface (Figure 5.11).

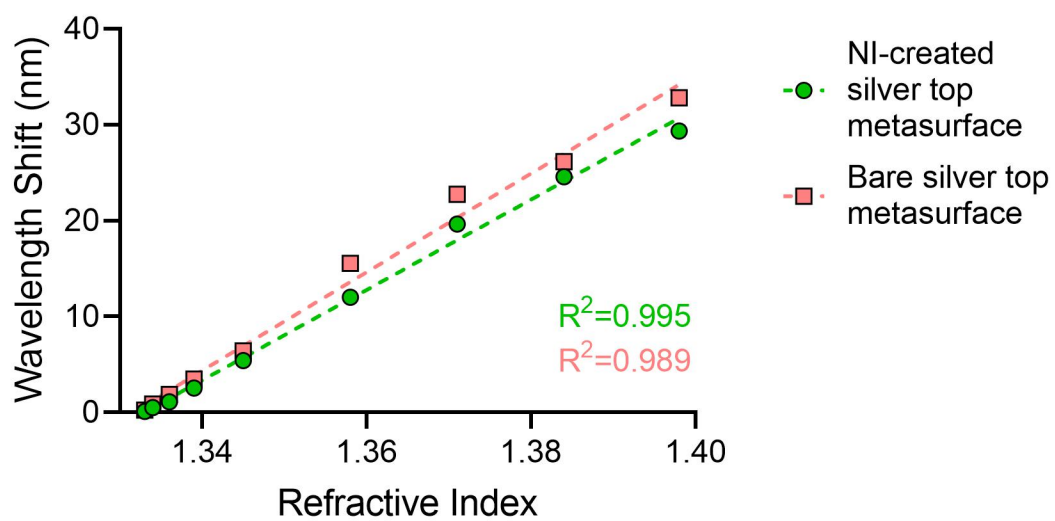


Figure 5.11: Comparison of the bare silver top and NI-formed silver top metasurfaces.

Chapter 6

Biomarker Detection

6.1 Surface Sensitivity

As aforementioned, the sensor sensitivity was considered with two concepts: (i) bulk sensitivity and (ii) surface sensitivity. The latter, surface sensitivity is also another applicable parameter for the plasmonic-based biosensing platforms than the bulk sensitivity since it measures a fraction of the electric field near the metal surface. In the case of biosensing, molecular binding occurs near the plasmonic layer, and only the RI of this confined region changes [187].

In this chapter, the surface sensitivity while applying surface chemistry and monitoring the binding of target molecules (a disease biomarker) was investigated on the sensor surfaces. Additionally, any effects caused by complex matrices were evaluated using biomarker (exosome)-spiking into artificial urine instead of PBS buffer. First, protein G was decorated on the surface in order to organize the orientation of antibodies (either anti-CD81 antibodies or anti-CD63 antibodies). Once the antibodies were immobilized, exosomes were applied to the sensors, and the binding analysis was monitored in real-time using our portable setup, thereby also assessing the performance of the platform and sensor.

6.2 Immobilization of Protein G to the Sensors

For the development of biosensors, surface functionalization is one of the crucial steps for specificity and sensitivity. A homogenous and well-oriented surface chemistry would potentially enhance the capture of target molecules. For antibody-based detection, their orientation is also substantial, and the Fab sections of antibodies would be in representing state for proper detection whereas the Fc part would be immobilized on the regarding domain of protein G on the sensor.

In this study, protein G was immobilized through a layer-by-layer surface chemistry, including 11-MUA and EDC/NHS linkers. 11-MUA contains a thiol end that binds metal (gold) surface, and its carboxylic end reacts with EDC/NHS linkers to create a succinimide end for protein binding. The success of binding protein G on the bare surface was characterized by the XPS in Chapter 4.

For the AuNP-integrated and NI-formed surfaces, the bare sensor were pre-modified with a positively charged PLL solution. Hereafter, we applied the layer-by-layer surface chemistry with 11-MUA and EDC/NHS linkers, and protein immobilization could not be achieved. Consequently, we directly applied protein G on the AuNP-integrated and NI-formed surfaces. In Figure 6.1, 100 $\mu\text{g}/\text{mL}$ of protein G was applied on the NI-formed surfaces, and real-time measurements were performed. The experiment started with generating a baseline with PBS for 300 seconds at a 10 $\mu\text{L}/\text{minutes}$ of flow rate. Followingly, 100 $\mu\text{g}/\text{mL}$ of protein G was supplied through the channel at a 10 $\mu\text{L}/\text{minutes}$ of flow rate for 10 minutes. Then, the syringe pump was stopped, and the samples in the channels were incubated for an hour. To remove unbound proteins, the sensor was washed with PBS for 5 minutes. The wavelength was shifted for 0.72 nm on the gold top surface and 0.56 nm on the silver top surface; therefore, successful protein G binding was achieved on both sensor (Figure 6.1).

a) Gold top surface

b) Silver top surface

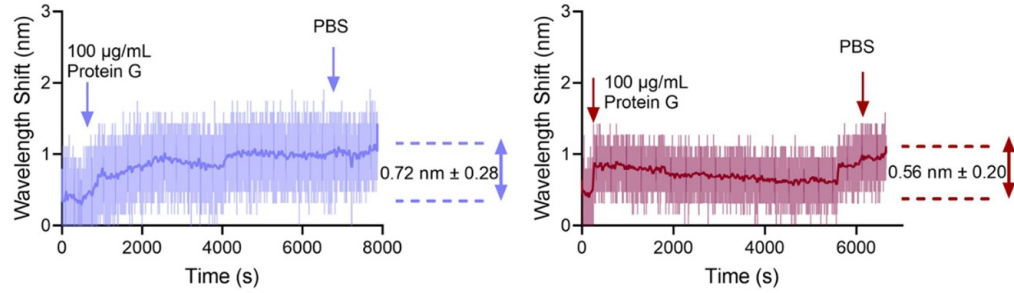


Figure 6.1: Immobilization of protein G ($100 \mu\text{g/mL}$) onto the NI-formed (a) gold top and (b) silver top surface.

6.3 Immobilization of Anti-CD81 Antibodies to the Surface

To demonstrate successful immobilization of anti-CD81 antibodies onto protein G was monitored in real-time. Until antibody immobilization, the surfaces were prepared as described in the earlier sections. (i) For the bare surfaces, the layer-by-layer surface chemistry (11-MUA – EDC/NHS – protein G) was performed, and as a final step, $50 \mu\text{g/mL}$ of anti-CD81 antibodies were introduced to the sensor and incubated for 3 hours at $+4^\circ\text{C}$. For the bare gold top surface, the immobilization of antibodies was characterized by contact angle and XPS in Chapter 4. (ii) For AuNP-integrated gold and silver top metasurface, only protein G was immobilized by pre-prepared surface directly (there was no earlier steps, i.e., 11-MUA – EDC/NHS). To monitor the successful binding of antibodies on protein G immobilized AuNP-integrated gold and silver top metasurfaces, optical measurements were performed in a real-time manner (Figure 6.2). The experiment started with generating a baseline with PBS for 300 seconds at a $10 \mu\text{l/minutes}$ of flow rate. Next, $50 \mu\text{g/mL}$ of anti-CD81 antibody was applied to the AuNP-integrated gold top metasurface through the channels at $10 \mu\text{l/minutes}$ of flow rate for 10 minutes. In this process, the syringe pump was stopped, and

the samples were incubated for an hour. To remove unbound molecules, PBS was applied for 5 minutes at the same flow rate. The same experimental protocol was repeated for AuNP-integrated silver top surface. The wavelength was shifted for 2.4 nm on the AuNP-integrated gold top metasurface and 1.87 nm on the AuNP-integrated silver top surface (Figure 6.2). Consequently, the binding of the antibodies was performed effectively. These results pointed the successful immobilizations of the protein G and anti-CD81 antibodies, and we continued to detect exosome (selected as a model biomarker for kidney disease) for the following sections.

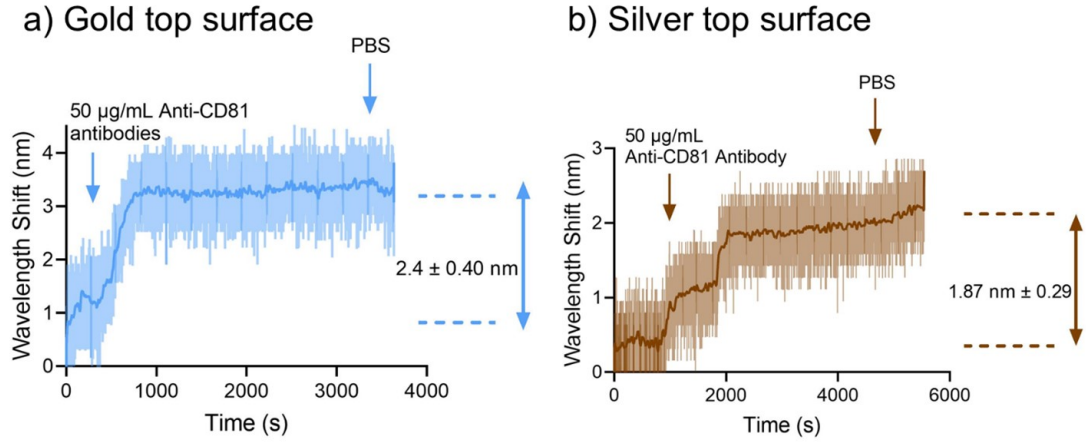


Figure 6.2: Immobilization of anti-CD81 antibodies ($50 \mu\text{g/mL}$) onto protein G-modified (a) AuNP-integrated gold top metasurface and (b) AuNP-integrated silver top metasurface.

6.4 Biomarker Detection

Biomarkers are described as the measurable diagnostic indicators to evaluate the risk of an abnormal condition such as disease [189]. They have a pivotal role in disease diagnosis from several sample sources, including blood or urine [190]. In this chapter, we selected exosomes as a model biomarker for kidney diseases since the recent studies have elucidated that exosomes hold huge potential for diagnosing kidney diseases early [163, 167]. To detect exosomes, four main steps

were present here: (i) fabricating a sensor surface, (ii) functionalization of the sensor, (iii) the isolation of the exosomes through a microfluidic chip, and (iv) detecting exosomes on the sensor. The details of each step was explained in the previous sections. Briefly, for the first step, gold and silver top metasurfaces were fabricated. Then, they were modified with 0.5 mg/mL of PLL. Later, the NI-formation and AuNP-integration were evaluated through bulk sensitivity studies (glycerol experiments).

In the second step, the bare sensor surfaces were modified with 11-MUA, EDC/NHS chemistry; then, protein G and anti-CD81 antibodies were immobilized on the sensor surface. The success of the surface chemistry was characterized by contact angle and XPS (described in Chapter 4). NI-formed and AuNP-integrated surfaces were directly functionalized with protein G and anti-CD81 antibodies. Their binding events to the surface were monitored with the optical setup and quantified as a wavelength shift. After the surface functionalization was successfully completed, the third step was performed.

As the third step, sample preparation was employed with exosome isolation and sampling. Exosomes were initially isolated from the medium of HEK293 cells using a microfluidic chip [8]. The concentration, diameter, and size distributions of exosomes were analyzed using NTA Instrument. The concentration of the stock solution was varying between 3×10^8 and 10^8 particles/mL. For the comparison of the signal enhancement, one sample concentration was set for each condition by diluting the stock solution with PBS to reach to 10^8 particles/mL. Six different sensor surfaces were prepared (the bare versions of gold top and silver top; AuNP-integrated gold and silver top; NI-formed gold and silver top surfaces), and 10^8 particles/mL exosomes were captured on each surface separately. All experiments were repeated three times. Spectroscopic measurements were performed with (i) bare metasurface, (ii) AuNP-integrated metasurface, and (iii) NI-formed metasurfaces, which were functionalized anti-CD81 antibodies (have an affinity to CD81 surface protein on exosomes). For the baseline, PBS was applied to the channel for 500 seconds, and then, the exosome sample (10^8 particles/mL) was supplied to the channel for 45 minutes at $10 \mu\text{L}/\text{minutes}$ of flow rate. PBS

was used to remove unbound particles, and the resulting wavelength shift was calculated. To imitate complex matrices, exosome stock solution was also diluted with artificial urine in order to reach 10^8 particles/mL.

6.4.1 The Bare Version of Metasurfaces

The first experimental set for exosome detection was performed with the bare versions of gold and silver top surfaces. To evaluate their performances, we measured plasmonic signals by tracking the second resonance peak (indicated resonance in Figure 5.3a-b) in real-time. The measurement setup was started with the recording background from the mirror (as described in Chapter 5), and then, PBS was applied to anti-CD81 antibody-immobilized channel to generate a baseline for 500 seconds. Afterward, samples (10^8 particles/mL) were injected into the channel for 45 minutes at $10\ \mu\text{L}/\text{minutes}$ of flow rate. To remove unbound molecules, PBS wash was performed for 5 minutes. The captured exosomes on the bare gold top surface were shifted to the wavelength of $1.27 \pm 0.18\ \text{nm}$ (Figure 6.3a). These experiments were repeated three times, and averaged wavelength shift was calculated as 0.94 ± 0.29 (Figure 6.4a).

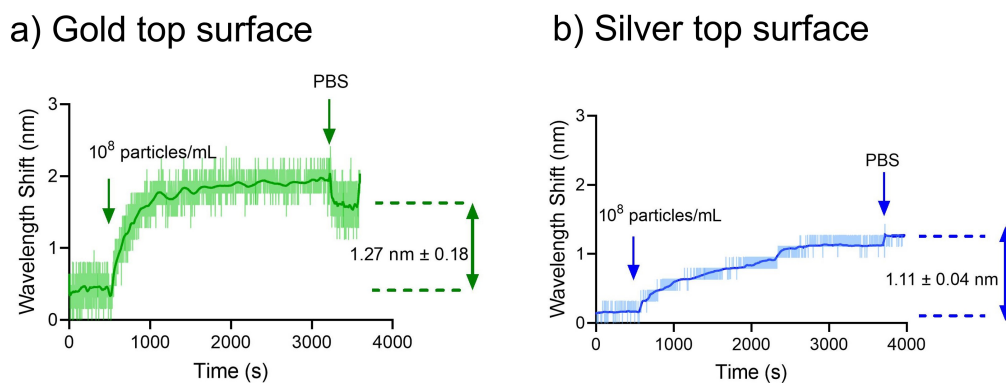


Figure 6.3: Assessing the plasmonic responses while capturing exosomes on anti-CD81 antibodies immobilized (a) bare gold top and (b) bare silver top metasurfaces.

The same experimental protocol was performed for the bare version of silver top surfaces, and we observed 1.11 ± 0.04 nm of wavelength shifts (Figure 6.4b). When the experiment was repeated three times, the average wavelength shift was quantified as 0.77 ± 0.34 nm. After normalizing the data (0-1), the signal of gold top surface was ~ 3.2 times higher than that of silver top surface (Figure 6.4b).

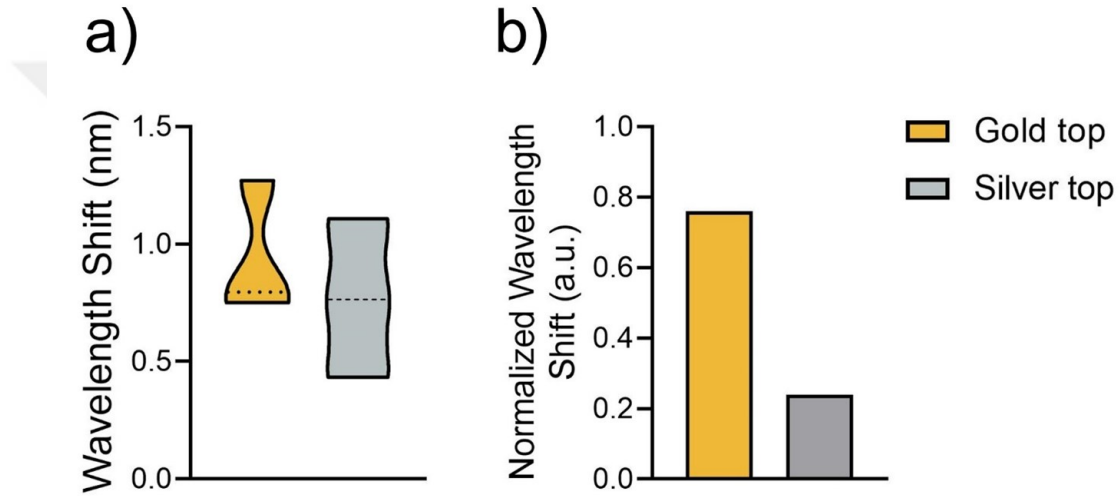


Figure 6.4: (a) Violin-box graph presents wavelength shifts after exosome captured on bare gold and silver top metasurfaces. The data was analyzed by performing a unpaired parametric t-test statistical analysis, and there was no statistical difference observed ($n=3$, $p>0.05$) (b) The normalized data (0-1) of wavelength shift is demonstrated after exosomes captured on bare gold and silver top metasurfaces.

6.4.2 AuNPs-Integrated Metasurfaces

The second experimental condition was to perform the exosome detection on AuNP-integrated versions of gold top and silver top metasurfaces. In the plasmonic-based biosensing platforms, signal enhancement can be achieved by constraining electromagnetic fields into small regions near the nanostructure surface, such as nanostructures with sharp corners or integrating nanoparticles [8]. The optical properties of a substrate can be tuned or manipulated by arranging

AuNPs three-dimensionally on the surface [191]. In this regard, AuNPs were integrated on the gold and silver top metasurfaces to enhance the signals and reach lower detection concentrations. The optimum AuNPs amount was determined in Chapter 5. After attaining optimum signals, the surface was functionalized with protein G without any pre-chemical treatment. 100 $\mu\text{g/mL}$ of protein G and 50 $\mu\text{g/mL}$ of anti-CD81 antibodies were directly applied to the AuNPs in an order as described in the previous sections. These two surfaces were employed to capture exosomes (same concentration and protocol for surface functionalization). Similar to the bare surface experiments, PBS was applied to anti-CD81 antibody-immobilized channel to create a baseline. Exosome solution (10^8 particles/mL) was applied into the channel for 45 minutes at 10 $\mu\text{L/minutes}$ of flow rate. To remove unbound molecules, a PBS-wash was performed for 5 minutes. While applying 108 particles/mL to AuNP-integrated versions of gold and silver top sensors, we observed ~ 1.82 nm and ~ 2.32 nm shifts, respectively (Figure 6.5a-b).

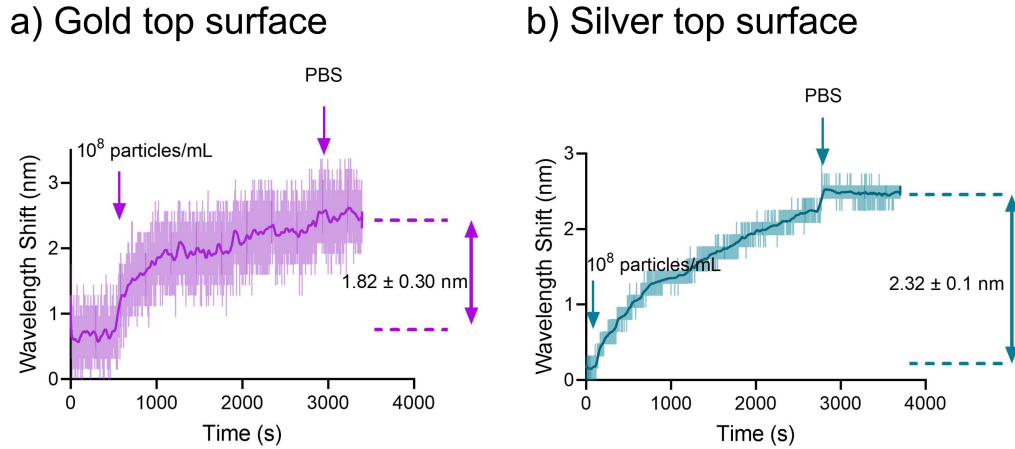


Figure 6.5: Evaluating the plasmonic responses while capturing exosomes on anti-CD81 antibodies immobilized (a) AuNPs-integrated gold top and (b) AuNPs-integrated silver top metasurfaces.

When the experiments were repeated three times, the average shift was calculated at 1.54 ± 0.52 nm for the AuNP-integrated version of gold top surface and 2.47 ± 1.49 nm for the AuNP-integrated version of silver top surface (Figure

6.6a). After normalizing the data (0-1), the case of silver top surface provided ~ 3.2 times higher signals compared to the gold counterpart (Figure 6.6b).

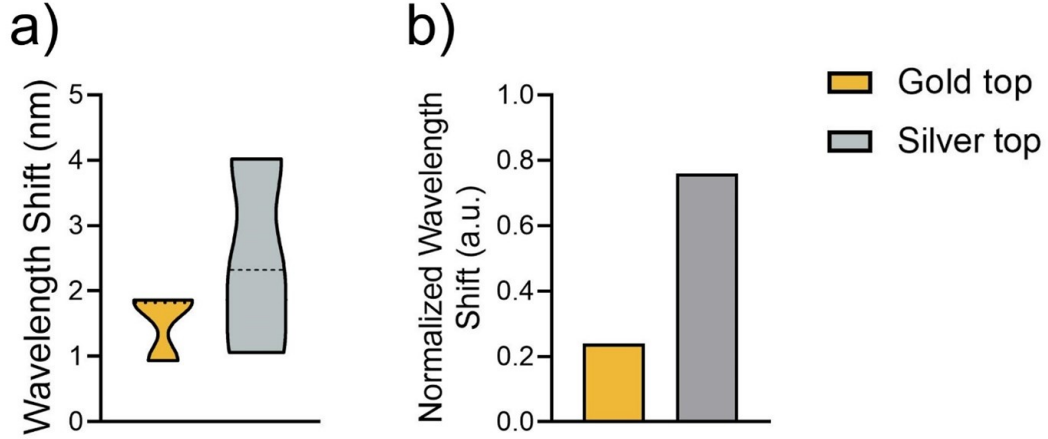


Figure 6.6: (a) Violin-box graph presents wavelength shifts after exosome captured on AuNP integrated gold and silver top metasurfaces. The data was analyzed by performing an unpaired parametric t-test statistical analysis, and there was no statistical difference observed ($n=3$, $p>0.05$) (b) The normalized data (0-1) of wavelength shift is shown after exosome captured on AuNP integrated gold and silver top metasurfaces.

6.4.3 NI-Formed Metasurfaces

In the concept of plasmonic biosensor, creating hot spots on the plasmonic suberates is a strategy for enhancing signals [59]. As the third experimental condition, NI was constructed on the nanoperiodic structure to enhance the sensitivity. In Chapter 5, the optimized parameters for NI-formation were defined considering to the bulk sensitivity. In this section, all NI-formed surfaces were prepared by 0.5 mg/mL of PLL-modified surface and 10 μ M of seeding for 5 minutes. Followingly, as described in the previous sections, 100 μ g/mL of protein G and 50 μ g/mL of anti-CD81 antibodies were directly applied to these surfaces. In the earlier sections, the binding of the protein G and anti-CD81 antibodies were confirmed through real-time monitoring on the sensor surface. In addition,

antibody immobilization and exosome capture on the sensor surface were monitored to validate exosome capture. For that purpose, an optimized NI-formed surface on a gold top metasurface was employed. Likewise, the experimental setup was started with the flow of PBS to create a baseline for 500 seconds at 10 $\mu\text{L}/\text{minutes}$ of flow rate. Afterward, 50 $\mu\text{g}/\text{mL}$ of anti-CD81 antibodies were injected into the channel 10 minutes flow and 45 minutes incubation (no flow). One-step of PBS-wash was performed to remove unbound molecules for 5 minutes at 10 $\mu\text{L}/\text{minutes}$ of flow rate. Similar to the exosome capture experiments, 10^8 particles/mL of exosomes were injected into the channel for 45 minutes at 10 $\mu\text{L}/\text{minutes}$ of flow rate. To remove unbound particles, another PBS-wash was employed, and each wavelength shift was calculated (Figure 6.7). Antibody immobilization shifted for 0.87 nm, and the exosome detection caused to 1.02 nm of wavelength shift.

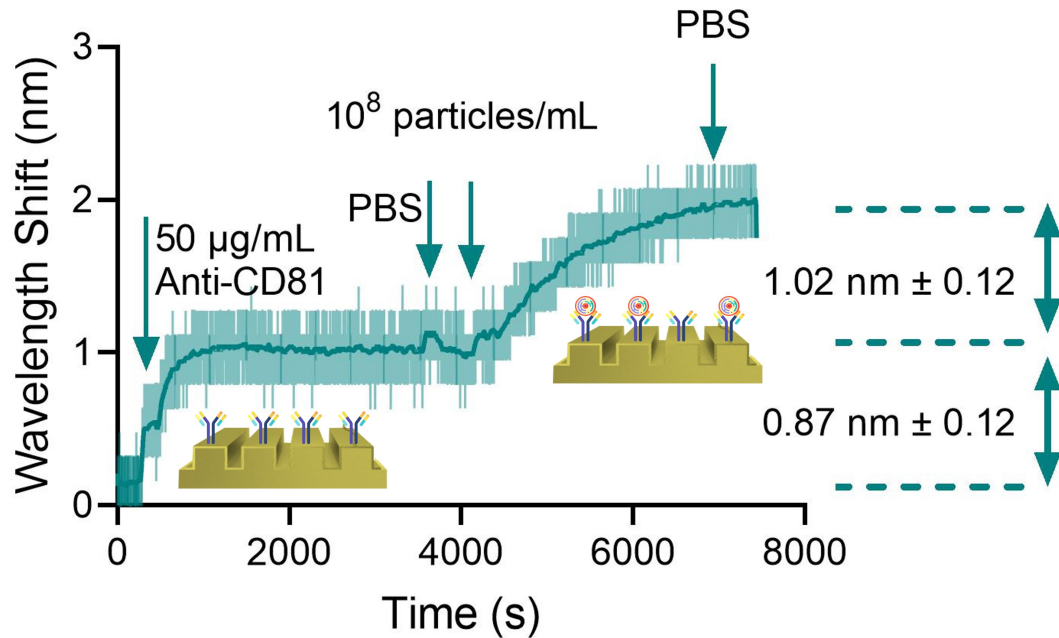


Figure 6.7: The real-time monitoring of anti-CD81 binding for 45 minutes of incubation and subsequently applying 10^8 particles/mL of exosomes onto NI-formed gold top metasurfaces.

As we confirmed the immobilization of antibodies to the sensor through protein G, we did not perform the real-time measurements. As the usual case, antibodies were incubated for 3 hours in the surface chemistry approach. Once the surface chemistry steps were completed, we measured the capture and detection of exosomes using the optical setup. Similar to the bare and AuNPs-integrated surfaces, PBS was flowed for 500 seconds to create a baseline. Then, 10^8 particles/mL exosomes were injected into the channel for 45 minutes at $10 \mu\text{L}/\text{minutes}$ of flow rate. To remove unbound particles, a PBS-wash was again performed. The wavelength shift was calculated by subtracting the first PBS wavelength from the second PBS wavelength. The wavelength shifts were calculated as 1.7 ± 0.31 nm and 0.43 ± 0.1 nm for gold and silver top surfaces, respectively (Figure 6.8a-b).

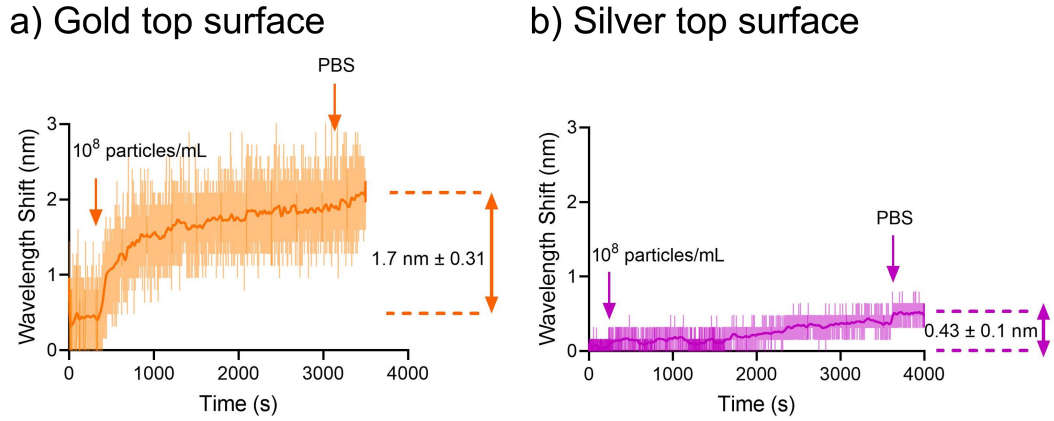


Figure 6.8: Evaluating exosome capture on (a) NI-formed gold top metasurface and (b) NI-formed silver top metasurfaces which are modified with anti-CD81 antibodies.

When we compare Figure 6.7 and Figure 6.8a, they have the same surface (NI-formed gold top metasurfaces) and same surface chemistry steps ($100 \mu\text{g}/\text{mL}$ of protein G and $50 \mu\text{g}/\text{mL}$ of anti-CD81 antibodies); however, wavelength shift was less than 60%. The main reason might be the the incubation time of the surface chemistries since anti-CD81 antibodies were incubated for only 45 minutes. On the other hand, in the usual case, they were incubated for 3 hours. When the experiments were repeated three times, the average shift was calculated at

1.27 ± 0.50 nm for the gold top surface and 0.42 ± 0.02 nm for the silver top surface, respectively (Figure 6.9a). After normalizing the data (0-1), the case of gold top surface provided ~ 3.2 times higher signals compared to the one of silver top surface (Figure 6.9b).

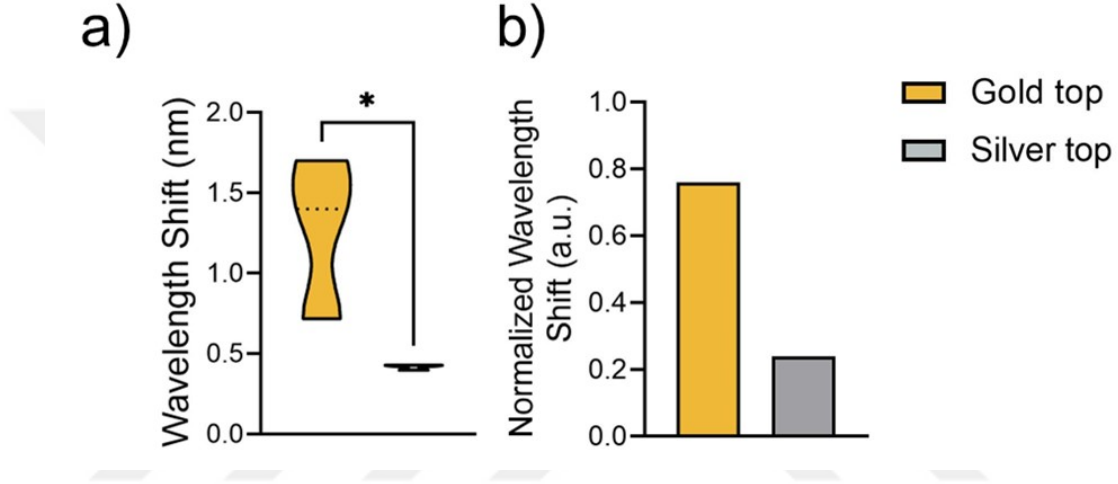


Figure 6.9: (a) Violin-box graph presents wavelength shifts after exosome captured on NI-formed gold and silver top metasurfaces. The data was assessed by performing an unpaired parametric t-test statistical analysis, and the statistical difference was shown with an asterisk ($n=3$, $p<0.05$). (b) The normalized data (0-1) of wavelength shift is presented after exosome captured on NI-formed gold and silver top metasurfaces.

Gold and silver top surfaces were evaluated among themselves to assess the enhancement of the fabricated metasurfaces. For instance, three versions of gold top metasurfaces ((i) bare, (ii) AuNPs-integrated, and (iii) NI-form metasurfaces) were further investigated. The wavelength shifts were calculated as ~ 1.27 , ~ 1.82 , and ~ 1.70 nm for one measurement on these versions (Figure 6.10). Once each experiment was repeated three times, averaged values were determined as 0.94 ± 0.29 nm, 1.54 ± 0.52 nm, and 1.27 ± 0.50 nm (Figure 6.11a). After normalizing the data (0-1) to compare the results with bare surface, we observed that more than 3.5 times in NI-formed surface, and 5.5 times signal enhancements in AuNP-integrated surfaces. In addition, AuNP-integration to gold top surface

enhanced the signal for ~ 1.6 times compared to NI-formed metasurfaces (Figure 6.11b).

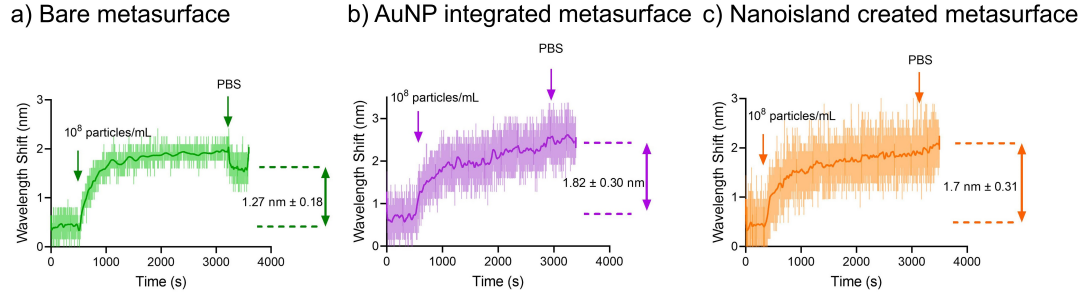


Figure 6.10: Assessing the plasmonic performance in real-time while capturing 10^8 particles/mL of exosomes on (a) the bare gold top, (b) AuNPs-integrated gold top, and (c) NI-formed gold top surface.

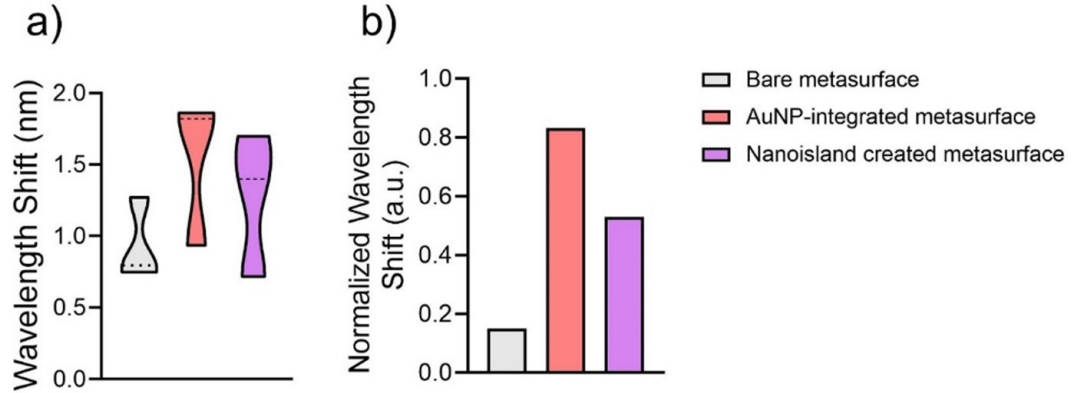


Figure 6.11: (a) Violin-box graph of the three measurements of each condition for gold top metasurfaces and (b) normalized data (0-1). The data was analyzed by performing a non-parametric Kruskal-Wallis statistical analysis, and there was no statistical difference observed ($n=3$, $p>0.05$).

Similarly, the wavelength shifts of (i) bare silver top surface, (ii) AuNPs-integrated silver top metasurface, and (iii) NI-formed silver top metasurfaces were examined further. The wavelength shifts were calculated as 1.11 nm, 2.32 nm, and 0.43 nm for one measurement of these surfaces, respectively (Figure 6.12). Once each experiment was repeated three times, averaged values were determined as

0.77 $\pm$ 0.34 nm, 2.47 $\pm$ 1.49 nm, and 0.42 $\pm$ 0.02 nm (Figure 6.13a). After normalizing the data (0-1), we observed that $\sim$ 2.6 times signal enhancements observed in AuNP-integrated surface compare to the bare version whereas NI-formed surface caused to $\sim$ 0.68 times reduction in the signal compared to the bare surface. In addition, AuNPs-integration to silver top metasurface enhanced the signal $\sim$ 3.8 times compared to NI-formed silver top metasurfaces (Figure 6.13b).

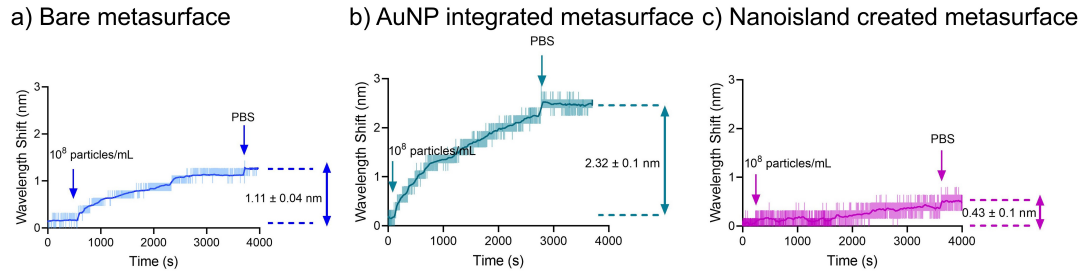


Figure 6.12: 10^8 particles/mL exosomes capture on bare (a) silver top (b) AuNPs-integrated silver top surface and (c) NI-formed silver top surface in real-time.

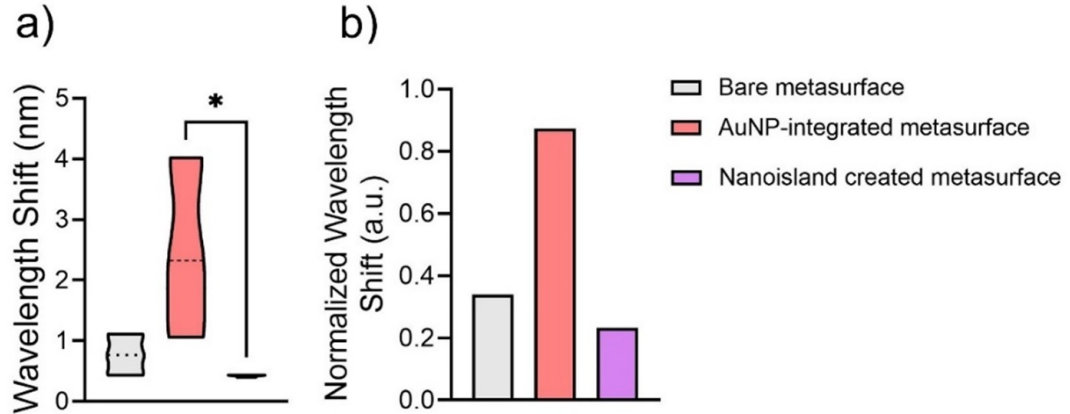


Figure 6.13: (a) Violin-box graph of the three measurements of each condition for silver top metasurfaces and (b) normalized data (0-1). The data was evaluated by performing a non-parametric Kruskal-Wallis statistical analysis, and the statistical difference was shown with an asterisk ($n=3$, $p<0.05$).

6.5 Anti-CD63 Antibody Experiment

The membrane of the exosome contains tetraspanin proteins—CD63, CD81, and CD9. In this chapter, most of the experiments were performed with anti-CD81 antibodies; however, to indicate versatility of this platform, anti-CD63 antibodies were also employed to capture exosomes in the optimum condition, i.e., AuNPs-integrated silver top metasurface.

As elaborated above, protein G was applied directly after the AuNP-integration to the silver top metasurface, and then, anti-CD63 antibody were immobilized. After that, 10^8 particles/mL of exosomes were introduced to the channels for 45 minutes at $10 \mu\text{L}/\text{minutes}$ of flow rate after creating a baseline with PBS.

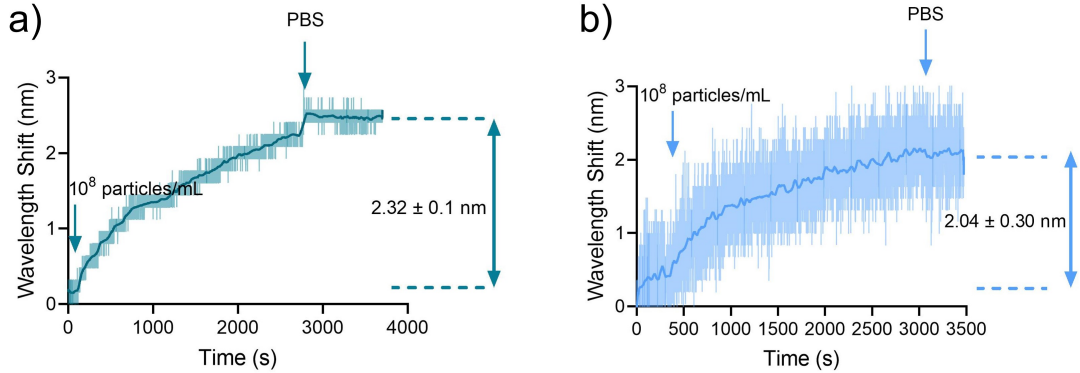


Figure 6.14: Evaluating the antibody capturing capability (anti-CD81 antibodies and anti-CD63 antibodies) for exosomes. In the experiments, 10^8 particles/mL of exosome were applied to (a) anti-CD81 antibody immobilized AuNPs-integrated silver top metasurface, and (b) anti-CD63 antibody immobilized the AuNPs-integrated silver top metasurface.

Briefly, the AuNP-integrated versions of silver top metasurface resulted in $2.47 \pm 1.49 \text{ nm}$ (average of three measurement) of wavelength shifts, respectively. Considering the wavelength shifts, anti-CD63 antibodies successfully captured the exosomes (2.04 nm shift), indicating the versatility of these sensors (Figure 6.14).

Therefore, these sensors would be easily adapted to detect the other biomarkers through the modification of surface chemistry by only changing the antibodies.

6.6 Artificial Urine Experiment

While developing a biosensor platform for target analytes, the first analysis was mainly carried out using physiological buffers (PBS buffer). In practice, end-users do not use these buffers, and they directly apply bodily fluids to the sensors and medical devices. Since bodily contain a wide variety of compounds such as cells, proteins, lipids and cell debris, these compounds could possibly alter specificity and sensitivity. Therefore, pre-clinical validation needs to be carried out before the transition of these products to be tested with clinical samples.

In this regard, the optimized conditions for gold and silver top surfaces were investigated with artificial urine to imitate the sample source for pre-clinical applications [192]. The highest wavelength shift was observed from AuNPs-integrated gold top metasurfaces and silver top metasurfaces in the previous experiment; therefore, artificial urine experiments were performed with these surfaces. The surfaces were prepared with 0.5 mg/mL of PLL modification, followed by 1:10 dilution of AuNPs were applied to the pre-modified surface, and the surface chemistry steps were performed with 100 $\mu\text{g/mL}$ of protein G and 50 $\mu\text{g/mL}$ of anti-CD81 antibodies. The only difference in exosome samples was that stock solutions were diluted with artificial urine instead of PBS buffer since urine samples are common for diagnosing kidney diseases.

The experiments were started with the PBS injection for 500 seconds, and then, 10^8 particles/mL of exosomes spiked in artificial urine were applied for 45 minutes at 10 $\mu\text{L/minutes}$ of flow rate. An sharp increase was observed when the sample was filled in the channel and this might be due to the ingredients in artificial urine. After 45 minutes, PBS was performed to remove unbound molecules. Once the PBS was filled to the channel, a sharp decrease was observed, pointing out that this sharp increase/decrease would be due to different

RI value of artificial urine compared to PBS. As a result, the wavelength shift was calculated as 1.30 nm for AuNPs-integrated gold top surface (Figure 6.15a). When the experiment was repeated three times, the average was determined as 1.15 ± 0.44 nm. The AuNPs-integrated silver top metasurface shifted 1.82 nm once 10^8 particles/mL of exosomes spiked in artificial urine was applied to the channel. Despite testing AuNPs-integrated silver top metasurface with artificial urine (3 different experiments), only one experiment provided negligible data compared to the other two experiments. The results of AuNPs-integrated silver top metasurface in artificial urine were comparable to the same experiments performed in PBS buffer (Figure 6.15b).

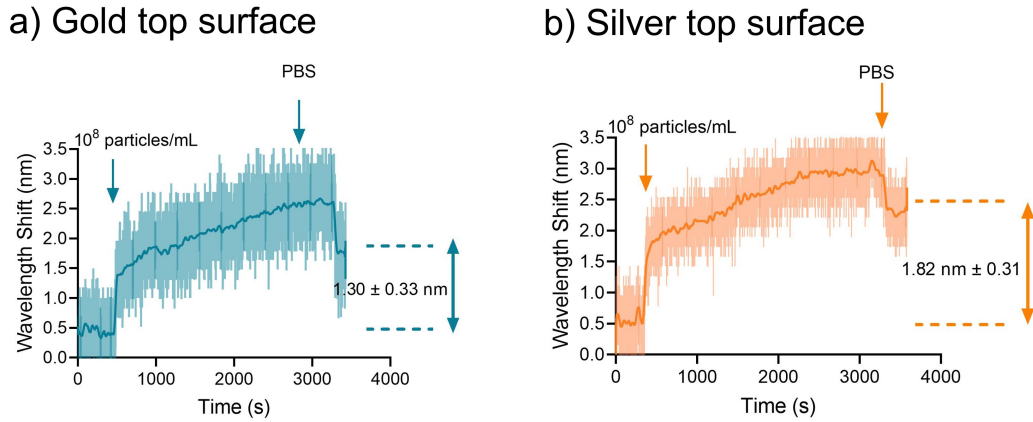


Figure 6.15: Exosomes (10^8 particles/mL) spiked in artificial urine were applied onto the AuNPs-integrated (a) gold top metasurfaces and (b) silver top metasurfaces.

The artificial urine experiments were performed with the bare versions of gold and silver top metasurfaces. In addition, we compared the capture of exosomes on bare sensors with the control groups (the urine solution lacking exosomes). Here, the bare gold top surfaces were functionalized with anti-CD81 antibodies through 11-MUA, EDC/NHS, and protein G. Then, 10^8 particles/mL of exosomes spiked in artificial urine was applied to the sensor. To understand any non-specific bindings, control sample (artificial urine without exosomes) was injected into the channel for 45 minutes, and the data was monitored in real-time for both gold and bare silver surfaces.

For the bare version of gold top surface, exosomes spiked in artificial urine caused to 0.43 nm of wavelength shift. When the control sample was applied, there were no shifts observed. Only sharp increase and decrease in the signals were observed due to the RI change (Figure 6.16).

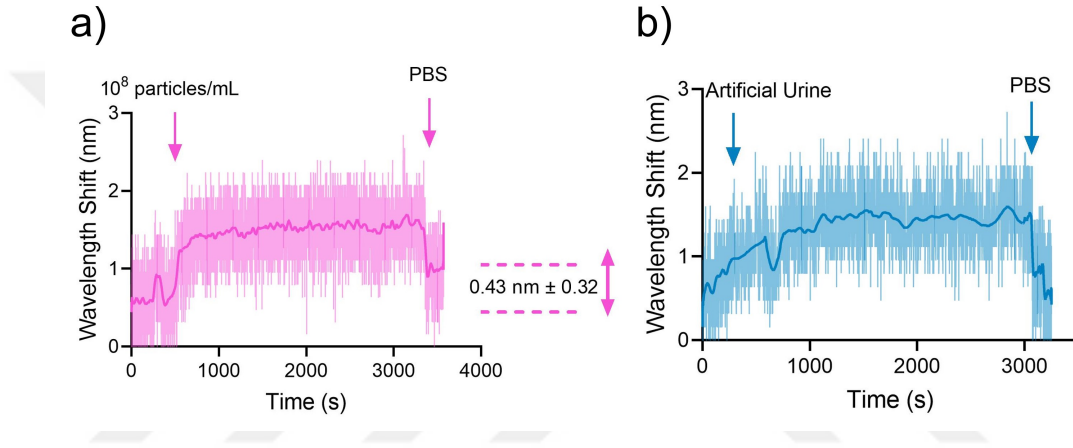


Figure 6.16: Exosomes (10^8 particles/mL) spiked in artificial urine were applied onto the bare gold top metasurfaces. (b) A control experimental set was performed while applying only artificial urine without exosomes to the same characteristics of metasurface.

For the bare version of silver top surface, exosomes spiked in artificial urine caused to 1.00 nm of wavelength shift, which was close to the same sensor version in PBS experiment. Likewise, the control sample did not cause any wavelength shifts, and similar, sharp increase and decrease in the signals were observed here as well (Figure 6.17). Overall, these results indicated that this platform achieved to detect exosomes in more complex solutions as a pre-clinical validation, and in the future, it would be ready for testing with real samples collected from patients in a specific disease model.

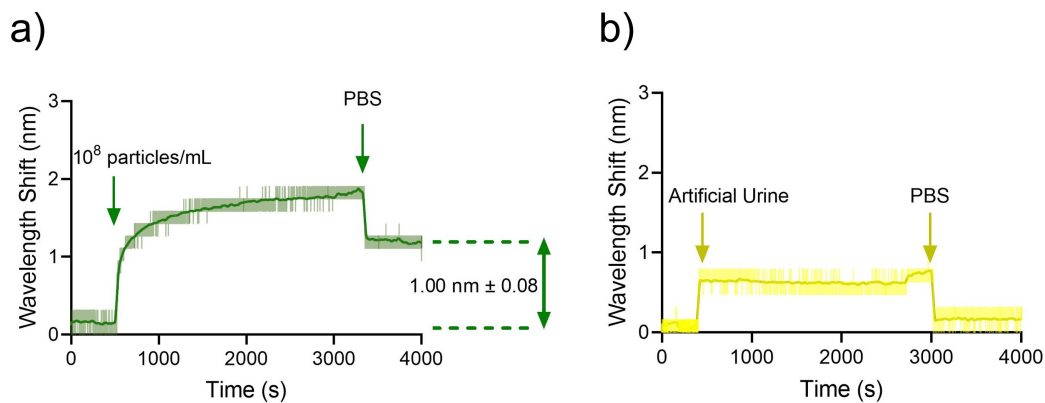


Figure 6.17: Exosomes (10^8 particles/mL) spiked in artificial urine were applied onto the bare silver top metasurfaces. (b) A control experimental set was performed while applying only artificial urine without exosomes to the same characteristics of metasurface.

Chapter 7

Conclusion and Future Prospects

In this study, we developed a metamaterial-based plasmonic sensor relying on a multi- or single layer of metal coating on a recycled, off-the-shelf optical disk, which was further modified with biomaterials, AuNPs, and NI formation. The nanograting structure was obtained from optical disks (DVDs), hence enabling a cost-effective and facile fabrication procedure compared to highly expensive fabrication strategies requiring sophisticated facilities and specialized personnel. Using optical disks also allows for large-scale and easy production of the sensors. In practice, there are also specialized and expensive nanopatterning methodologies including lithographic processes, hot embossing, and replica molding, off-the-shelf optical disk have already nanograting structure intrinsically, and they cost for only \$0.90 - \$1.50. Considering bulk purchases, this cost would be decreased to pennies.

As elaborated above, optical disks were turned into sensors by coating multi (titanium, gold, and silver) and monolayer (silver) of metals using thermal evaporation. As a next step, these metasurfaces were coated with PLL as the biomaterial, which enabled to integrate AuNPs, as well as assisted to form NI on the sensor. Interestingly, PLL was vital substrate to generate NI. On the other hand, the formation of NI did not occur without the PLL layer, and the resonance signal was not enhanced as depicted in Figure 5.4.

One of the most important points of this study was the optimization of all these procedures. The first part was to evaluate the PLL concentrations along with the seeding solution. To find the optimized PLL concentration, a fixed concentration of seeding solution ($10\text{ }\mu\text{M}$) was applied on 0.05, 0.1, 0.5 and 1 mg/mL of PLL-modified surface. The surfaces were benchmarked by injecting various glycerol solutions (1%- 70% of glycerol having RI values ranging from 1.333 to 1.428 RI) since plasmonic-based sensors exploit a signal response when refractive index is changed. The attained results showed that 0.5 mg/mL of PLL concentration resulted in higher S value (546.42 nm/RIU) compared to 1 mg/mL of PLL-modified surface (S value is 500.957 nm/RIU), 0.1 mg/mL of PLL-modified surface (S value is 542.505 nm/RIU) and 0.05 mg/mL of PLL-modified surface (S value is 515.9779 nm/RIU) and therefore, the remaining parts of procedure was continued with 0.5 mg/mL of PLL concentration.

The second optimization process was performed to evaluate the number of AuNPs on the nanograting structure. Initially, we realized the excess number of AuNPs damaged the nanograting structure of the sensor, and this resulted in no-signal. The optimized AuNP number was achieved while diluting the main stock in 1:10 (v:v) ratio with water. The exact amount of the AuNPs could not be determined. The OD value of AuNP stock (1:10 diluted) would be a criterion to understand the number of the particles (0.200 OD (a.u.) at 526 nm which is suitable for 30 nm AuNPs). However, batch-to-batch variations would be considered and needed to be optimized for precise use of AuNPs on the sensors. The surfaces were then benchmarked with various glycerol solutions (1%- 70% of glycerol having RI values ranging from 1.333 to 1.428 RI). Here, we observed that 1:10 dilution of AuNPs resulted in higher S values; for instance, 496.84 nm/RIU for gold top and 515.75 nm/RIU for silver top. Hence, the remaining part of the procedure was continued with these sensors. For biomarker detection, these surfaces were functionalized with the anti-CD81 antibodies, which have an affinity to CD81 (one of the tetraspanins on exosomes). The RI change occurred when the target analytes were attached to the surface, and binding was quantified in terms of the wavelength shift. The average wavelength shift was determined as

1.54 nm and 2.47 nm for gold top and silver top metasurfaces (Table 7.1). A linear correlation was observed when the bulk and surface sensitivity of the sensors were compared. The silver top surface had higher sensitivity in both bulk and surface sensitivity.

The third optimization process was performed for NI formation on the nanograting structure through a seeding reaction (chemical growth method), and thereby, the reaction needs to be stopped at some point. If excessive amount of NI was formed on the nanograting structure, the signal was lost as indicated in Figure 5.8b. In this regard, a variety of seeding solutions were assessed, and 10 μ M concentration was found as an optimum condition while benchmarking the sensor in that condition with various glycerol solutions (1%- 70%). For biomarker detection, these surfaces were functionalized with the anti-CD81 antibodies, and the average wavelength shift was calculated as 1.27 nm and 0.42 nm for NI-formed versions of gold top and silver top (Table 7.1).

Table 7.1: The summary of wavelength shifts collected on the metasurfaces while applying 10^8 particles/mL of exosomes.

Surface Material	Bare	AuNPs-integrated	NI-formed
Gold	0.94 nm $\pm$ 0.29 nm	1.54 nm $\pm$ 0.52 nm	1.27 nm $\pm$ 0.50 nm
Silver	0.77 nm $\pm$ 0.34 nm	2.47 nm $\pm$ 1.49 nm	0.42 nm $\pm$ 0.2 nm

Comparing the results with bare versions of gold and silver top metasurfaces, AuNP-integrated and NI-formed versions resulted in more wavelength shifts, pointing out an increase in the sensitivity. The highest sensitivity was observed while employing AuNPs-integrated silver top surface and then, the second one was AuNPs-integrated gold top surface. Therefore, these surfaces would be used for more low concentrations of target analytes and/or for the other biomarkers. For instance, in the case of Alzheimer’s Disease, biomarkers such as A β 42, phosphorylated tau (p-tau) proteins, and total tau (t-tau) proteins are very low in

blood; and therefore, more sensitive platforms are necessary for their detection in clinical applications [193].

Moreover, this study presented a novel kidney biomarker (exosome), which was isolated an innovative microfluidic chip platform consisting of two filter membranes. Briefly, exosomes carry essential information, which enhances their applications in diverse fields, including disease diagnostics, drug delivery, and theranostics applications. Besides gigantic potential as a biomarker, their isolation has limitations since they are very small particles and broadly varying in size (30 to 100 nm) [166]. In practice, ultracentrifugation is one of the conventional methods for their isolation; however, this equipment is highly costly, bulk, highly-complicated for common use and it results in low yield (Inci, 2022; Shirejini ve Inci, 2022). Here, we have introduced a filter-integrated microfluidic chip, which isolated exosomes in a size manner. The fabrication of microfluidic chip is simple (10 minutes to construct one device) and inexpensive ($\sim 2\$$). The concept of the device utilized a step-wise filtering; therefore, two different pore-sized membranes were employed: (i) 200 nm for larger particles and (i) 50 nm for smaller particles like exosomes. The sample was supplied from the side of 200 nm of pore-sized membrane and collected from the side of 50 nm of pore-sized membrane. The working volumes or reusability of the chip were also investigated as a part of another study [8]. The operating volume would be increased by connecting the devices or antifouling strategies would enhance reusability by reducing cake formation [8]. Moreover, this device would be used for different-sized particles isolation by changing the pore sizes on the membranes. After exosomes were collected from the outlet, NTA analysis determined the size and concentration of these particles. Then, exosomes were introduced to the sensor to evaluate their detection in a quantitative manner (wavelength shifts). The highest wavelength shift was observed while employing AuNPs-integrated silver top. Considering the signal-wise, the others in order were AuNPs-integrated gold top, NI-formed gold top, bare version of gold top, bare version of silver top, and the least wavelength shift was observed on NI-formed silver surface. SEM images also indicated that NI was not homogenously created on the silver stop surface.

Moreover, we have developed a portable setup coupled with a commercial spectrometer, which was easily operated with our in-house software for both real-time and end-measurements. The size of this portable setup is 7.5 cm x 5 cm x 3 cm, and it is likely a palm-size device that can be moved to anywhere for the operation. The optical components are also easy-to-access, and they are basic lenses, polarizers, and aperture, etc. The setup is combined with the z stage to arrange the distance of incoming light to the surface, only tuning the height arrangement of the signal. The sample holder of the setup is flexible, and it allows to place different size of sensors and free movement on any place on the sensor.

In the literature, a variety of biosensing platforms have been developed to detect exosomes for AKI diagnosis. Typically, these strategies employ antibodies against tetraspanins (CD9, CD63, CD81, and CD82) receptors of exosomes. Particularly, cancer-related exosomes mostly carry some protein biomarkers from the originated cells, such as CEA, EpCAM, HER2, IGFR, LMP1, MUC18, and PSMA. In addition, several biosensing approaches (e.g., colorimetry, fluorescence, surface plasmon resonance, surface-enhanced Raman scattering, and electrochemical methods) have been utilized to detect AKI-related biomarkers [194]. In the table 7.2, we have comprehensively demonstrated different exosome detection platforms along with the origin of the isolated exosomes, sensor types, biorecognition elements, their advantages, and disadvantages. Most of these studies are centralized on plasmonic biosensing concept.

On the other hand, a study indicates the formation of NI on the gold-coated surface, which is fabricated via deposition techniques [195]. The main challenge in that study is that the surface was not specified for a target molecule. The protocol requires to mix anti-CD9 antibodies with the isolated exosomes, and then, this mixture is applied to an NI-formed surface. Antibody-exosomes complexes are attached to the surface. Therefore, there is a high potential for non-specific binding from a complex sample (e.g., blood, serum or urine). In our study, we decorated our NI-formed structures with antibodies which were specific to the exosome surface markers, and detected them in physiological buffers (PBS) and artificial urine samples (pre-clinical model). In both cases, we provided specific

detection of exosomes without any noticeable signal background caused by the non-specific interactions. In addition, our strategy provided the formation of NI on the sensor within only 5 minutes whereas the other study requires ~ 3 hours [195].

Moreover, there are some studies utilizing commercial products for exosome isolation or they require expensive and complicated traditional techniques, which highly limits their clinical utility for further processes. As shown in our earlier study [8], we isolated exosomes via a facile-to-use microfluidic platform that requires only sampling through a syringe pump. To detect exosomes, we recycled the optical DVDs for sensor fabrication, and boosted the sensitivity via either the formation of NIs and the integration of AuNPs to the gold top or silver top metasurfaces. For pre-clinical applications, AuNP-integrated gold and silver top surfaces were evaluated with exosomes spiked in artificial urine, and the performance was promising since we did not see a notable amount of background signals while testing the sensors with control groups (artificial urine without exosomes). The wavelength shifts were also comparable with the those tested with exosomes spiked in PBS.

Table 7.2: Existing exosome detection platforms and comparison of their features with the platform presented in this thesis.

Origin of Exosome	Sensor Type	Biorecognition element	Detection Strategy	Exosome isolation method	Detection of limit	Advantages	Disadvantages	Refs
Culture Medium (MCF-7)	Electrochemical sensor	Aptamer	Hybridization chain reaction on screen-printed electrode	Exospin kit (Promega)	5×10^3 particles/mL	Label free	•Enzymatic reaction with specified conditions •No detection in complex fluids •Long assay time (~ 2 h)	[196]
Culture Medium (NSCLC) and serum	Plasmonic sensor	•Anti-EGFR antibodies •anti-PD-L1 Antibodies	Refractive indexes change on the Au film	Thermo Fisher Scientific (4478359) isolation kit	2×10^{10} exosomes/mL	Validation with human samples	Complex setup	[197]
Plasma from healthy individuals	Feld-effect transistor (gFET)	Anti-CD63 antibodies	Electrical measurement from Titanium, gold gates and a graphene layer	Bought from Hansa Biomedical	$0.1 \mu\text{g/mL}$	Label-free detection	No detection in complex fluids	[198]

Culture medium (m A549)	Plasmonic sensor	Anti-EGFR antibodies	Plasmonic interferometer array (PIA)	Not reported	3.86×10^8 exosomes/mL	Smart-phone integration	•Microscopy-based optical setup •Expensive tools	[199]
Culture medium, Blood, urine and serum samples from mouse	Plasmonic biosensor	Anti-CD9 antibodies	LSPR interferometer	Ultracentrifugation	0.194 $\mu\text{g/mL}$	Validation with blood serum, and urine with mouse model	•Deposition techniques for Nanoislands •No detection in complex fluids	[195]
Culture Medium and clinical sample	Plasmonic sensor	•Anti-CD9 antibodies •Anti-HER2 antibody	custom-made SPR platform	Total Exosome isolation reagent (Life Technologies)	2070 exosomes/ μL	Detection of cancerous exosome	•Sandwich assay •Complex component-based detection platform	[200]
Culture medium (HMC-1.2)	Plasmonic biosensor	anti-CD63 antibodies	Biacore 2000 instrument for SPR measurements	differential centrifugation with a filtration step	4 $\mu\text{g/mL}$	•Commercial SPR instrument •Label free detection	Benchtop instrument (Not portable setup)	[201]

This study Culture Medium (HEK 293)	Plasmonic sensor	•Anti-CD81 antibodies •Anti-CD63 antibodies	Refractive indexes change on the gold and silver nanograting contained surface	Filter-based microfluidic device	1×10^8 exosomes/mL	•Detection in complex fluids •Label free detection •Cheap sensor fabrication •Portable measurement setup	Lack of validation with patient samples	—
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Overall, advances in biosensing technologies provide substantial potential for disease diagnosis, and focus further deployments to make them as POCT devices. In this regard, plasmonic sensing is one of the most applied modalities to detect and quantify targets varying in size and composition—from proteins to exosomes and cells. In this thesis, we presented a facile and inexpensive strategy to fabricate nano-periodic metasurfaces along with plasmonic fashion. As the plasmonic behavior and utility of materials would be different, the top of plastic surfaces was coated with either gold or silver. In order to boost plasmonic characteristics, we further immobilized biomaterials as adlayers to seed a chemical reaction for the formation of NI, and bind AuNPs. All these manipulations and tuning efforts improved analytical performance of metasurfaces, especially the sensitivity aspect. In addition, we benchmarked these sensor surfaces successfully while detecting exosomes spiked in artificial urine as a pre-clinical testing. All the plasmonic signals were monitored in real-time by using our palm-sized platform and an in-house software—coupled with our commercial spectrometer, and all these components (sensors, optical components, spectrometer, and in-house software) worked seamlessly for detecting the exosomes. In sum, we achieved to (i) recycle off-the-shelf optical disks into metamaterial biosensors; (ii) develop a

portable setup for detecting minute concentrations of exosomes; (iii) open a new horizon for exosome detection considering highlighting features such as inexpensive ($< \$1.5$ per chip), facile-use (two steps: sampling and washing), and rapid turnaround (45 minutes per test); (iv) resolve the cost- and complexity-related challenges in deploying new biosensing strategies into the POC settings, and all these advancements make our platform one step closer to the technology that would be employed in clinic or research laboratories, even at bed-side in near future.

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Appendix A

Appendix

A.1 Declaration of Copyright Clearance Agreements

Reference Number	Licence Supplier	Licence Number
[6]	American Chemical Society	Permission not required
[7]	MDPI	Permission not required
[8]	American Chemical Society	Permission not required