

Development of Metal and Metal Oxide Nanoparticles and Investigation of Their Potential Applications

by

Özge Çavuşlar

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of Their Potential Applications**

Koç University

Graduate School of Sciences and Engineering

This is to certify that I have examined this copy of a doctoral dissertation by

Özge Çavuşlar

and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

Committee Members:

Assoc. Prof. Havva Yağcı Acar

Prof. Mehmet Kaya

Prof. Duygu Avcı Semiz

Assoc. Prof. Mehmet Burçin Ünlü

Assist. Prof. Sarp Kaya

Date: _____



Dedicated to my family..

ABSTRACT

In the past few decades, nanoparticles have gained great importance in research and industry due to their adjustable chemical, physical, and biological properties. Gold nanoparticles (GNPs) and superparamagnetic iron oxide nanoparticles (SPIONs) are the two most studied and most attractive structures. These nanoparticles have been used in a variety of applications such as catalysis, sensors, energy, and medicine for therapeutic and imaging purposes.

In this thesis, we aimed to develop extensive experience in gold nanoparticle synthesis and functionalization to control the particle size, stability, absorbance characteristics and surface chemistry to build a nanoparticle-based toolbox to be used in different applications from medicine to catalysis. For that purpose, a broad portfolio of functional gold nanoparticles by using different reducing and coating materials was produced. Cationic GNPs with polyethylenimine (PEI) is one of the target structures of this thesis due to its potential in gene delivery and catalysis. Anionic GNPs is another target structure for its potential in medicine.

In the field of SPIONs, sulfonic acid functionalized, small and stable SPIONs were produced as potential nanoparticles for a variety of purposes from targeted drug delivery and imaging to photo thermal therapy.

In the second chapter, synthesis of small, cationic GNPs by the direct reduction of auric acid in a non-reducing solvent, water, with branched polyethylenimine (bpi) in a broad pH range (3.0-9.0) was described. Basic pH, which was studied for the first time, emerged as a favourable condition to achieve good reducing power and surface passivation simultaneously, providing small particles (hydrodynamic size ca 6 nm) with enhanced long-term stability and sharper surface plasmon resonance peak (SPR). This synthetic method produces colloidal GNPs with bPEI in a broad molecular

weight range (0.6, 1.8, 10, 25kDa). Molecular weight (MW) did not influence the crystal size much but the hydrodynamic size and the stability.

This synthetic approach was adopted to GNPs produced by linear PEI (IPEI) in the third chapter. IPEI is accepted as more biocompatible than bPEI and in reduction of auric acid and stabilization of nanoparticles expected to behave differently since it lacks primary amines. Small, stable cationic GNPs were prepared for the first time with IPEI in water. Protonation and deprotonation of IPEI is a valuable variable for reduction, surface adsorption and stability. Impact of the PEI/Au ratio, polymer molecular weight (2.5 and 25 kDa), reaction pH (3.5 to 10), method of IPEI dissolution and post-synthetic pH on particle properties were studied. Finally, two different purification methods were evaluated to prevent the aggregation of GNPs. Protonation of PEI is needed for the synthesis and long-term stability of colloidal IPEI/GNPs regardless of the polymer MW. The best is to dissolve IPEI by protonation and to clean GNPs via controlled centrifugal precipitation. MW did not influence the hydrodynamic size, stability or particle shape, but low MW IPEI provided faceted particles. This simple one-pot synthesis of small, stable cationic GNPs in water is a valuable, simple alternative for producing new cationic GNPs with even low molecular weight IPEI.

In the fourth chapter, anionic gold nanoparticles were synthesized by a modified single-phase Brust-Schiffrin method by using 3-mercaptopropionic acid as the coating and NaBH_4 as a reducing agent. Those nanoparticles were primarily prepared as a biocompatible nanocarrier for enhanced transport of polyclonal Immunoglobulin G (IgG) across blood-brain barrier (BBB) in sepsis treatment. These GNPs were decorated with glucose to pass BBB and IgG was conjugated to GNPs with acid cleavable hydrazone linkages. Synthetic parameters for glucose and polyclonal IgG conjugation to GNPs were thoroughly investigated. Since IgG was polyclonal, it had a wide range of isoelectric point (pH 6.3-8.9) making the optimization of reaction pH and order of functionalization quite challenging. Additionally, understanding the

appropriate working environment with IgG was another challenge due to the bacterial attack.

In the fifth chapter, synthesis of reproducible, functional, and stable gold nanoparticles with absorbance in the visible and near-infra red (NIR) region were described. Relatively new, gold goldsulfide nanostructures with functional, stabilizing coatings were developed for the first time and their utility in drug delivery and photothermal therapy were investigated.

In the last chapter, a novel, green, one-pot aqueous synthesis method of sulfonic acid functionalized SPIONs were developed by utilizing the co-precipitation method and in-situ coating with (trihydroxysilyl)-1-propanesulfonic acid. The conventional synthesis method involves organic solvent. The new aqueous synthesis method described here is more suitable for biomedical applications. The proposed nanoparticle can be used in both catalysis and targeted combined therapies by loading cationic targeting molecules, drugs, and photosensitizers.

ÖZETÇE

Son yıllarda, araştırma-geliştirme ve endüstriyel alanlarında, özgün kimyasal, fiziksel ve biyolojik özellikleri nedeniyle metal bazlı nanomalzemelerin kullanımı büyük önem kazanmıştır. Altın nanoparçacıkları (ANP) ve süper paramanyetik demir oksit nanoparçacıkları (SPION) en çok çalışılan ve en ilgi çeken yapılardır. Her iki nanoparçacık, kataliz, sensör, enerji ve sağlık alanında terapötik ve görüntüleme amaçlı gibi çeşitli uygulamalarda kullanılmaktadır.

Bu tez çalışmasında, tıptan katalizöre kadar farklı uygulamalarda kullanılacak nanoparçacık bazlı bir alet kutusu oluşturmak için partikül büyüklüğü, kararlılığı ve absorbans özellikleri; ayrıca yüzey kimyasını kontrol etmek için altın nanoparçacık sentezi ve fonksiyonlandırılması üzerine kapsamlı bir araştırma yapılmasını amaçladık. Bu amaçla, farklı indirgeyici ve kaplama malzemeleri kullanarak geniş bir fonksiyonel altın nanoparçacık portföyü oluşturulmuştur. Polietilenimin (PEI) kaplı katyonik ANP'ler, gen taşınımı ve kataliz potansiyeli nedeniyle bu tezin temel konularından biridir. Anyonik ANP'ler tıptaki potansiyeli için başka bir ana başlığı kapmasamaktadır.

SPION alanında ise, sülfonik asit fonksiyonel, küçük ve kararlı SPION'lar, hedefli ilaç taşınımı ve görüntülemekten, fototermal terapiye kadar çeşitli alanlarda kullanılması amacı ile üretilmiştir.

İkinci bölümde, aurik asidin su içerisinde dalı polietilenimin ile geniş bir pH aralığında doğrudan indirgenmesiyle küçük, katyonik altın nanoparçacıkları (ANP) sentezlenmesi incelenmiştir. Bazik pH ortamında PEI kaplı ANP ilk kez çalışılmıştır. Bazik pH'da PEI'nin hem daya iyi bir indirgeyici olması, hem de yüzey pasivasyonu elde edilmesini sağlamıştır. Böylece daha uzun süre stabilizeye sahip ve daha keskin yüzey plasmon bandına sahip, hidrodinamik boyutu yaklaşık 6 nm olan ANPler sen-

tezlenmiştir. Bu sentez yöntemi ile farklı moleküler ağırlığa sahip (0.6, 1.8, 10, 25kDa) dallı PEI'ler ile koloidal ANP'ler üretilmiştir. Moleküler ağırlık, kristal boyutunu çok etkilememiş ancak hidrodinamik boyutu ve kararlılığı etkilemiştir.

Üçüncü bölümde de benzer parametreler lineer PEI (lPEI) kullanılarak incelenmiştir. lPEI, bPEIye göre daha biyouyumlu olarak kabul edilir. lPEI yapısında bPEIye göre primer amin grubu bulunmadığı için aurik asidin indirgenmesini ve nanoparçacıkların kararlılığını etkileyen faktörlerin farklı olması beklenmektedir. Küçük, kararlı katyonik ANPler su içerisinde lPEI ile ilk kez sentezlenmiştir. lPEI'nin protonlanması ve deprotonasyonu, indirgeme, yüzey adsorpsiyonu ve parçacık kararlılığı için değerli bir değişkendir. PEI / Au oranı, polimer molekül ağırlığı (2.5 ve 25 kDa), reaksiyon pH'ı (3.5 ila 10), lPEI'nin çözünme metodu ve sentez sonrası pH'ın parçacık özellikleri üzerindeki etkisi incelenmiştir. Son olarak, ANP'lerin toplanmasını önlemek için iki farklı saflaştırma yöntemi değerlendirilmiştir. Polimerin moleküler ağırlığından bağımsız olarak, koloidal lPEI-GNP sentezi ve uzun vadeli kararlılığı için lPEI'nin protonasyonuna ihtiyaç vardır. lPEI-GNP sentezi ve kararlılığı için en iyi yöntem lPEI protone ederek çözmek ve kontrollü santrifüj çökeltmesi ile temizlemektir. Moleküler ağırlık hidrodinamik boyutu, kararlılığı veya parçacık şeklini etkilememiştir, ancak düşük moleküler ağırlığına sahip olan lPEIler fasetlenmiş partiküllerin oluşmasını sağlamıştır. Çalışılan metot, küçük kararlı katyonik ANPlerin düşük moleküler ağırlığa sahip PEI'ler kullanılarak dahi tek seferde su içerisinde sentezlenmesine olanak sağladığı için değerli ve kolay bir alternatif sentez yöntemi olmuştur.

Dördüncü bölümde, anyonik altın nanoparçacıkları, kaplama olarak 3MPA ve indirgeyici bir ajan olan NaBH_4 kullanılarak modifiye edilmiş tek fazlı Brust-Schiffrin metodu ile sentezlenmiştir. Bu nanoparçacıklar öncelikle, sepsis tedavisinde kan-beyin bariyeri (KBB) boyunca poliklonal Immunoglobulin G'nin (IgG) daha iyi taşınması için biyolojik olarak uyumlu bir nanotaşıyıcı olarak hazırlanmıştır. Bu GNP'ler, KBB'yi geçmek için glikoz ile dekore edilmiş ve IgG, asitle ayrılabilir hidrazon bağlantıları ile GNP'lere konjuge edilmiştir. Bu deneyler sırasında, glikoz ve poliklonal IgG kon-

jugasyon parametreleri derinlemesine incelenmiştir. Kullanılan IgG poliklonal olduğu için, geniş bir izoelektrik nokta aralığına (pH 6.3-8.9) sahiptir. Bu sebeple doğru reaksiyon sırasını ve IgGye uygun doğru pH aralığında konjugasyon deneylerinin optimize edilmesi zaman almıştır. Ek olarak, poliklonal IgGye bakteriyel atakları engellemek için uygun çalışma ortamını anlamak başka bir zorluk olmuştur.

Beşinci bölümde, fonksiyonel, kararlı, görünür ve yakın-kızıl ötesi (NIR) bölgede absorpsiyonu olan ANP'leri sentezi çalışılmıştır. Nispeten yeni olan altın-altın sülfid nanoyapıları, fonksiyonelliğini ve kararlılığını arttırmak amacıyla ilk kez kaplama malzemeleri kullanılarak sentezlenmiştir. Geliştirilen bu parçacıkların, ilaç taşınımı ve fototermal terapi potansiyelleri incelenmiştir.

Son bölümde, yeni, çevreci ve tek basamakta sentezlenen sülfonik asit fonksiyonel SPION sentez yöntemi geliştirilmiştir. Bunun için kopresipitasyon yöntemi ve in situ (trihidroksisilil) -l-propanesülfonik asit kaplama olarak kullanılarak sülfonik asit fonksiyonel SPIONlar sentezlenmiştir. Geleneksel sentez methodu organik çözücü içerdiği için, yeni ve su ortamında yapılan sentez metodu özellikle biyomedikal uygulamalar için daha uygundur. Önerilen nanoparçacık hem katalizde hem de katyonik hedefleme molekülleri, ilaçlar ve ışığa duyarlılaştırıcılar yüklenerek hedefli kombine tedavilerde kullanılabilir.

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NOMENCLATURE

3MPA	3-mercaptopropionic
BBB	Blood brain barrier
BE	Binding energy
bPEI	Branched polyethylenimine
BSM	Brust-Schiffrin method
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DI	Deionized
DIC	Differential interference contrast
DLS	Dynamic Light Scattering
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
EDC	Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediamine tetraacetic acid
EDX	Electron Dispersive X-ray
EE	Encapsulation efficiency
FA	Folic acid
FBS	Fetal Bovine Serum
FTIR	Fourier Transform Infrared Spectra
GFB	Green Fluorescent Protein
GNC	Gold nanoclusters

GNP	Gold nanoparticles
GGS	Gold-gold sulfide
HAuCl ₄	Auric acid
HeLa	Human Cervical Carcinoma
HEK	293T Human embryonic kidney cell
HR	High resolution
ICP-MS	Inductively Coupled Plasma-Mass Spectrometer
ICU	Intensive care units
ITC	Isothermal Titration Calorimetry
LE	Loading efficiency
IPEI	Linear polyethylenimine
LSPR	Localized surface plasmon resonance
MB	Methylene blue
MDA-MB-231	Human breast carcinoma
MO	Methyl orange
MW	Molecular weight
MTT	Thiazolyl blue tetrazolium bromide
NaBH ₄	Sodium borohydride
NaIO ₄	Sodium periodate
NIR	Near-infrared
PBS	Phosphate buffered saline
PDI	Polydispersity index
PDT	Photodynamic therapy
PEG	Polyethylene glycol
PTT	Photothermal therapy
RT	Room temperature
SPION	Superparamagnetic iron oxide

Sulfo-NHS

TEM

TGA

UV

VCR

XPS

XRD

N-hydroxysulfosuccinimide

Transmission electron spectroscopy

Thermal gravimetric analysis

Ultra violet

Vincristine

X-ray photoelectron spectroscopy

X-ray diffraction

Chapter 1

INTRODUCTION

1.1 *Gold Nanoparticles*

Gold nanoparticles (GNPs) have been around for a very long time. In ancient times gold was used in coloring of glasses. The Lycurgus Cup seen in Figure 1.1 which was made in the 5th to the 4th century BC is one of the greatest example to the early use of metal nanoparticles in coloring.



Figure 1.1: The Lycurgus Cup, externally (left) and internally (right) illuminated. [1]

The glass of the cup consists of well-dispersed silver 66.2%, 31.2% gold, and 2.6% copper nanoparticles with sizes up to 100 nm. Therefore, its color changes as the location of the light changes due to reflected and transmitted light. [14] The scientific history of colloidal gold has begun with Faraday in 1857. [15] He studied for the first time the preparation and properties of colloidal gold and its interaction with light.

Since that time, GNPs attract researchers from different fields due to unique size and shape dependent optical and electronic properties coupled with inert nature, high molar absorption coefficient and very high chemical and physical stability.[16, 17]

Colloidal GNPs have a unique tunable absorption band in the visible region which is called as *localized surface plasmon resonance (LSPR)*. The phenomenon behind the LSPR band is that when the light at certain wavelength interact with GNPs it causes a collective coherent oscillation of the conduction band electrons in the same direction of the incident electric field which generates strong absorption and scattering as shown in Figure 1.2A. [2, 18] Plasmon is a coherently displaced electron cloud from its equilibrium position around a lattice of positively charged ions.[19] In bulk, plasmons cannot be excited by incident light, but, at the nanoscale, where conduction band electrons are confined, and where surface/volume ratio is increased, surface plasmons can be excited. Indeed, these electron oscillations may induce an electric field larger than the one of the incident light. According to the Mie theory, particle size, shape, surface composition and the dielectric constant of the local environment affect the SPR band intensity and the wavelength (Figure 1.2B).[20] Hence, position, intensity, and shape of LSPR band of metallic nanoparticles are indicative of particle shape, size and size distribution. In addition, LSPR band plays an important role in monitoring the colloidal stability of GNPs. Spherical, small GNPs ($\approx 10\text{-}20\text{nm}$) have absorbance at $\approx 520\text{nm}$ with a wine color. As the aggregation occurs light cannot polarize electrons sufficiently. Therefore, absorbance spectrum shows a red shift, the LSPR band becomes weaker and shifts to $\approx 530\text{nm}$ and the color of GNPs turn into deep purple. Eventually, GNPs precipitate upon excessive aggregation of primary particles. [2, 18, 3]

Producing colloidally stable GNPs is a challenge and also a key requirement especially for biological applications due to the interactions between GNPs and drugs or biological fluids require stable nanoparticles. Stabilization of nanoparticles may be achieved a electrostatic, steric or electrosteric interactions as shown in Figure 1.3 [3] Electrostatic (Van der Waals) interactions are simply attractive or repulsive inter-

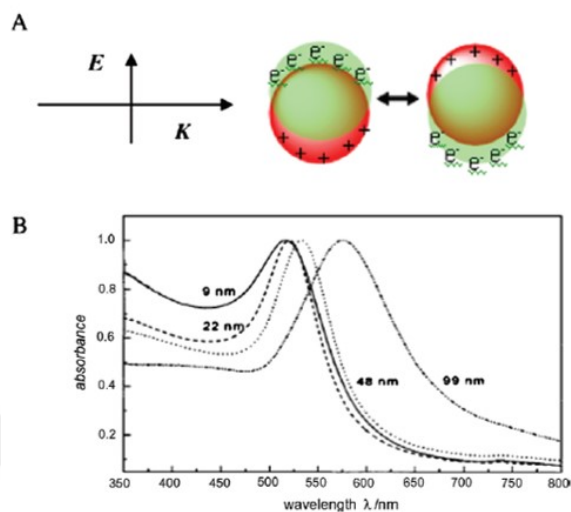


Figure 1.2: (A) Schematic illustration of surface plasmon resonance in plasmonic nanoparticles. (B) Absorbance spectra of gold nanoparticles in different sizes. [2]

actions between any charged molecules. Electrostatic interactions play an important role on conserving colloidal stability of GNP coated with short ligands such as citrate or 3-mercaptopropionic acid (3MPA). As the charge repulsion increases, the stability of GNP increases. Electrostatic interactions are easily affected from salt addition due to the quenching of electrical double layer. The charge of nanoparticles may be estimated by zeta potential (mV). Unless there are other types of interactions, particles are usually considered unstable between -30 and +30mV zeta potential due to lack of sufficient charge repulsion.[21] Utilizing polymers as a capping agent such as polyethylene glycol(PEG) and poly(N-isopropylacrylamide) (PNIPAM) produces stable GNPs.[22, 23, 24] When placed in an appropriate solvent, polymeric chains attain a conformation which favors the optimum distance from the nanoparticle surface where the configurational entropy is the lowest. Nanoparticles approaching each other usually closer than twice the thickness of the coating layer would make polymers lose conformational entropy which would cause repulsion of nanoparticles providing resistance to aggregation, in another word, colloidal stabilization of nanoparticles. Indeed, long hydrocarbon chains, such as dodecane thiol, does induce steric stabilization, as

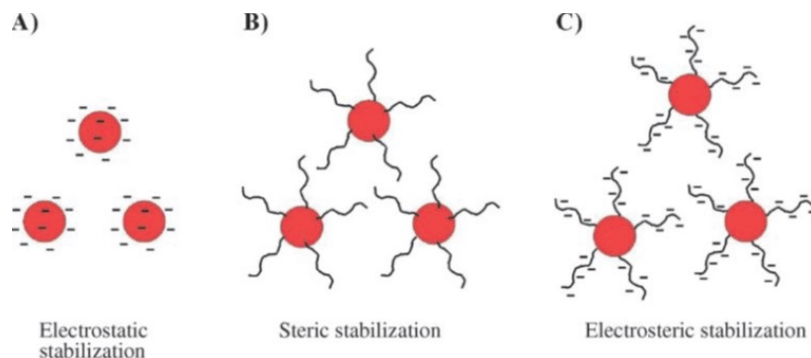


Figure 1.3: Illustration of interactions between GNPs (A)electrostatic, (B) steric, (C) electrosteric interactions [3]

well. Unlike the electrostatic interaction, steric interactions are more resistant to salt addition. Yet, the molecular weight and surface graft density affect the stability. Additionally, anionic and cationic polymers have both electrostatic and steric interactions due to charged grafted polymer chains. This type of interactions are defined as electrosteric interactions. Since electrosteric interactions are the combination of both electrostatic and steric interactions, they are affected from the large number of parameters such as pH, dielectric properties, ionic strength, graft density and the type of the surrounding medium.[25, 26]

GNPs are usually stabilized with small molecules, preferentially thiolated ones, or functional polymers. In the synthesis of GNPs from auric acid, often reducing agents such as NaBH_4 , $\text{Na}_3\text{-citrate}$ and amine functional molecules/polymers. [27, 28] During the synthesis, Au^{+3} is reduced to Au^{+1} and Au^0 , and the capping agent provides the stabilization and functionalization by adsorbing on the growing nanoparticle surface. Additionally, there are examples of using polymers and dendrimers as both reducing and capping agents in the synthesis of GNPs. [29]

Turkevich method and Brust-Schiffrin method are the most common synthesis techniques to produce GNPs. Both methods generate negatively charged particles. Turkevich method, the most widely used procedure to synthesize colloidal GNPs, produces large hydrophilic particles (8-120 nm) in aqueous media. Controlling the size of

GNPs depends on changing the molar ratio of HAuCl_4 , reaction pH and temperature as shown in Figure 1.4A. [4, 30, 31, 32] Citrate-capped GNPs are usually subjected to ligand exchange with an appropriate coating material, such as 2-mercaptoethanol (2MEA) or 3-mercaptopropionic acid (3MPA), suitable for the desired application of GNPs. Although widely used, this method usually causes aggregation of GNPs (1.4 (B-C)). [5, 33]

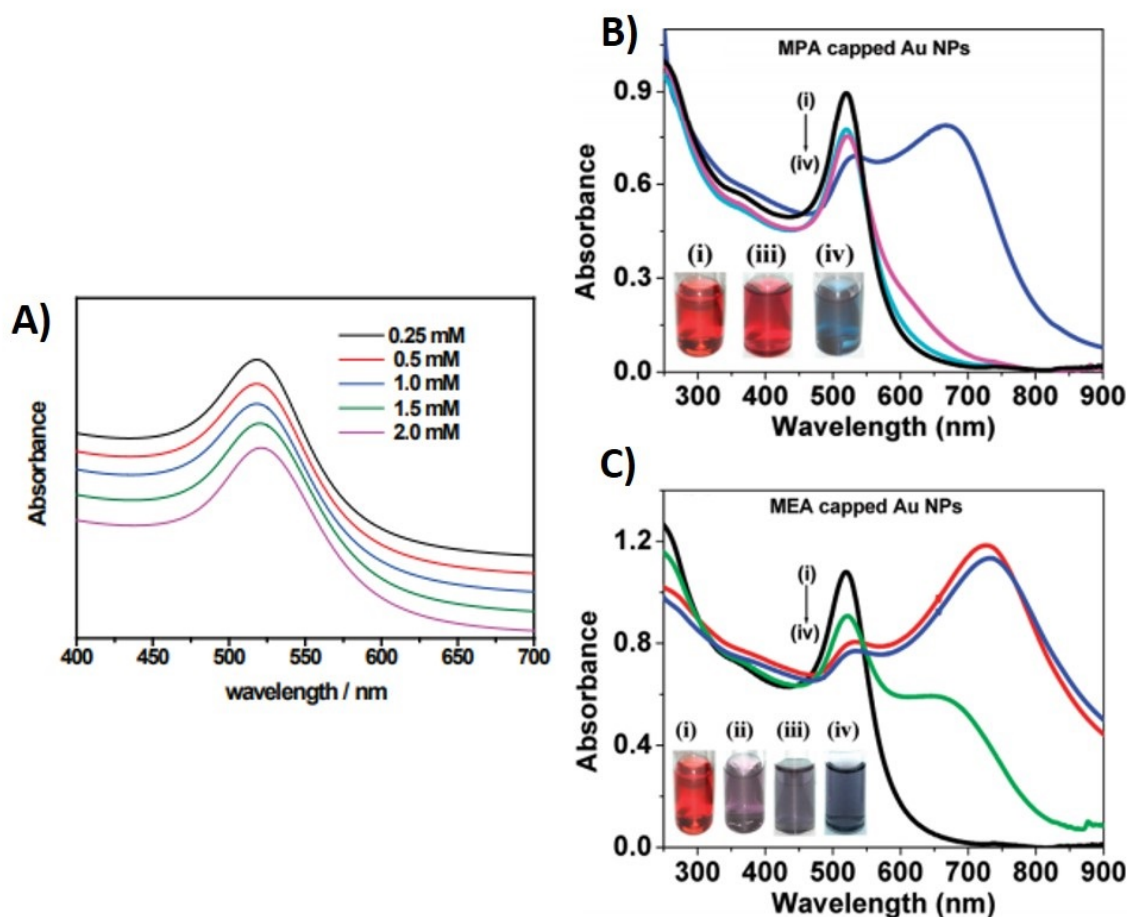


Figure 1.4: Absorbance spectrum of citrate-capped GNPs (A) with different HAuCl_4 concentrations, [4] (B-C) ligand exchanged with different concentrations of MPA and MEA. [5]

On the other hand, Brust-Schiffrin method (BSM) produces stable gold nanoclusters (GNCs) with 1-3 nm size.[34] The BSM was first published as a two-phase reduc-

tion method. Briefly, auric acid was first dissolved in water then transferred to toluene by using tetraoctylammonium bromide as a phase-transfer reagent and was reduced with aqueous sodium borohydride in the presence of dodecanethiol as the capping agent. A year later, they have published a single phase reduction method by using p-mercaptophenol as capping agent. In this method, auric acid and p-mercaptophenol were mixed in methanol, and aqueous sodium borohydride was added carefully under vigorous stirring. Small gold nanoclusters (GNCs) with sizes about 2nm were produced.[35] Thiolate protected gold nanoparticles can be dried and redispersed in solvents with no aggregation due to the strong covalent bond between Au and S.[34] Ever since, single phase BSM is widely used to produce small, stable GNCs by using 3-mercaptopropionic acid, glutathione and other thiolated ligands as capping agents. [6, 36, 37, 38] The schematic representation of the single phase BSM is given in the Figure 1.5.

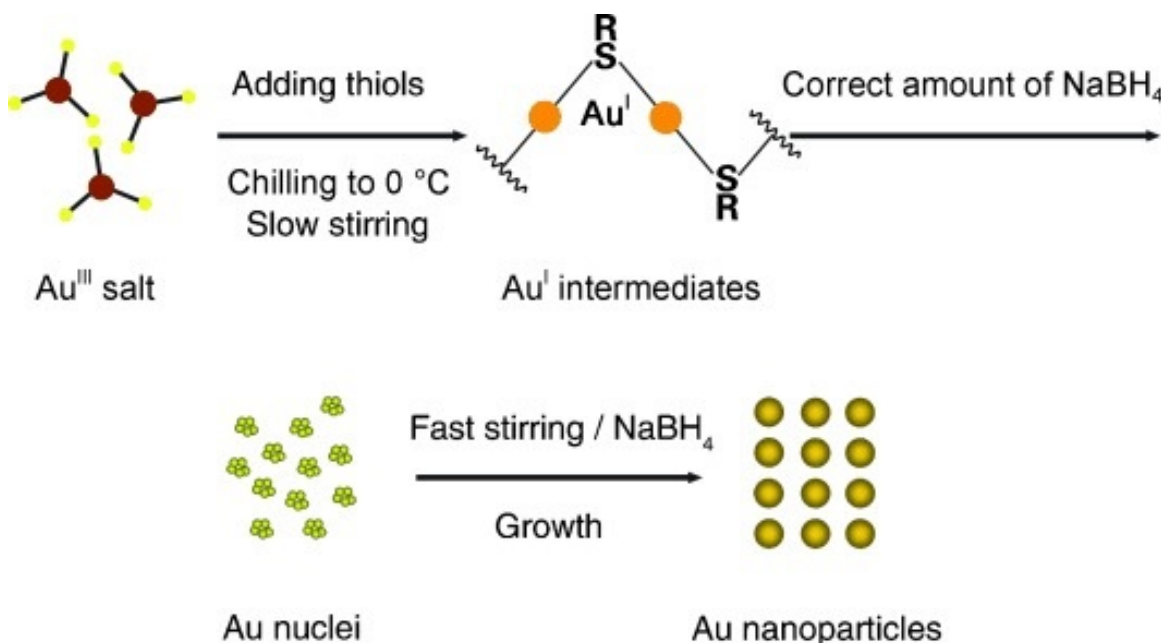


Figure 1.5: Illustration of the single phase Brust-Schiffrin method.[6]

In the production of GNPs with different surface functionality, NaBH₄ is the most preferred reducing agent in one pot room temperature (RT) synthesis in the presence

of a capping agent. The capping agents such as small molecules, peptides, proteins or polymers can be chosen according to the desired application.[39, 40, 41, 42]

1.1.1 NIR-absorbing GNPs

GNPs with tunable near-infrared (NIR) absorbance attracts researchers for several years. NIR wavelengths (650-900 nm) can penetrate into soft tissues up to 10 cm and are not harmful. [43] Additionally, between 700-950 nm, water is transparent and absorbance of NIR light by tissue/blood constituents such as hemoglobin is minimal.[44] Therefore, variety of gold nanoparticles were synthesized with NIR absorbance. In some studies, gold nanoparticles were synthesized as spherical nanoparticles with at LSPR band at 520nm and then aggregated with added salt and pH change to shift the absorbance to longer wavelengths. [45, 46] In other studies, non-spherical gold nanoparticles, such as nanorods, nanocages, nanostars, nanoshells and nanoplates, were synthesized to create second LSPR band in the NIR region. [47, 48, 49, 50, 51] However, these methods are quite difficult to repeat and costly.

Additionally, an interesting composition, gold-gold sulfide (GGS) nanoparticles, were reported with LSPR at 520nm and at 600-900 nm region. GGSs were published by Zhou et al. for the first time in 1994. [52] In 1997, Averitt et al. published a study on understanding the optical properties of GGS nanoparticles. [53] Both studies suggested that GGSs are Au coated Au₂S core/shell structures. Since then, several publications with an effort to understand the structure of GGS nanoparticles and their possible applications were released. Schwartzberg et al. were able to mimic the earlier synthetic procedure and characterized the produced nanoparticles in detail with transmission electron spectroscopy (TEM) and electron energy loss spectroscopy (EELS). [54] Based on the these analyses, they refused the core/shell structure and showed that GGSs are indeed aggregated particles of various shapes (rods , triangles) with sizes between 5 to 50 nm. EELS analysis indicated that only a thin layer of sulfur was visible on the surface of GNPs.

Considering these results, they proposed to name these particles as gold nanopar-

ticle aggregates (GNAs). They suggested that the strong interparticle interactions of these aggregated particles produce the LSPR band in NIR region. On the other hand, Mikhlin et al. proposed that nanoplates were creating the NIR-plasmon resonance.[55] They had created seed mediated growth of GGS nanoparticles which reduced the nanoplates formation. Additionally, the NIR-band intensity was decreased, and the peak was blue-shifted. They also studied the temperature effect on the particle formation. They point out that nanoplate formation is enhanced at 0°C, thus the NIR-band shifts to longer wavelengths. On the contrary, at 50°C, appreciable icosahedral nanoparticle formation takes place as well as nanoplate formation. Yet, the NIR-band shifts to shorter wavelengths. Even though GGSs absorb light in the NIR region, their stability is very poor, 3 days to 1 week due to lack of coating material, limiting their practical use. [55, 56]

Overall, GGS nanoparticles offer a great potential for NIR absorbance but requires stable nanoparticles and better understanding of its nature and synthetic requirements.

1.1.2 Applications of GNPs

GNPs with unique size and shape dependent optical and electronic properties added to chemical stability and biocompatibility excessively studied in catalysis, sensing, conducting materials, non-linear optics, nano-electronics, medicine, etc. (Figure 1.6). [16, 17, 57, 58, 59] In medicine, GNPs hold a great potential and exploited as diagnostic and therapeutic agents in addition to its use in biosensors and drug/gene delivery. [60, 61, 62]

Biomedical Applications

GNPs are under heavy investigation in both diagnostic and therapeutic applications (Figure 1.7). [63] GNPs have been widely used in dark field microscopy imaging of cancer cells due to intense scattering properties.[64] The scattering of ≈ 60 nm GNPs is approximately 100-fold stronger than the emission of a fluorescein molecule.[65]

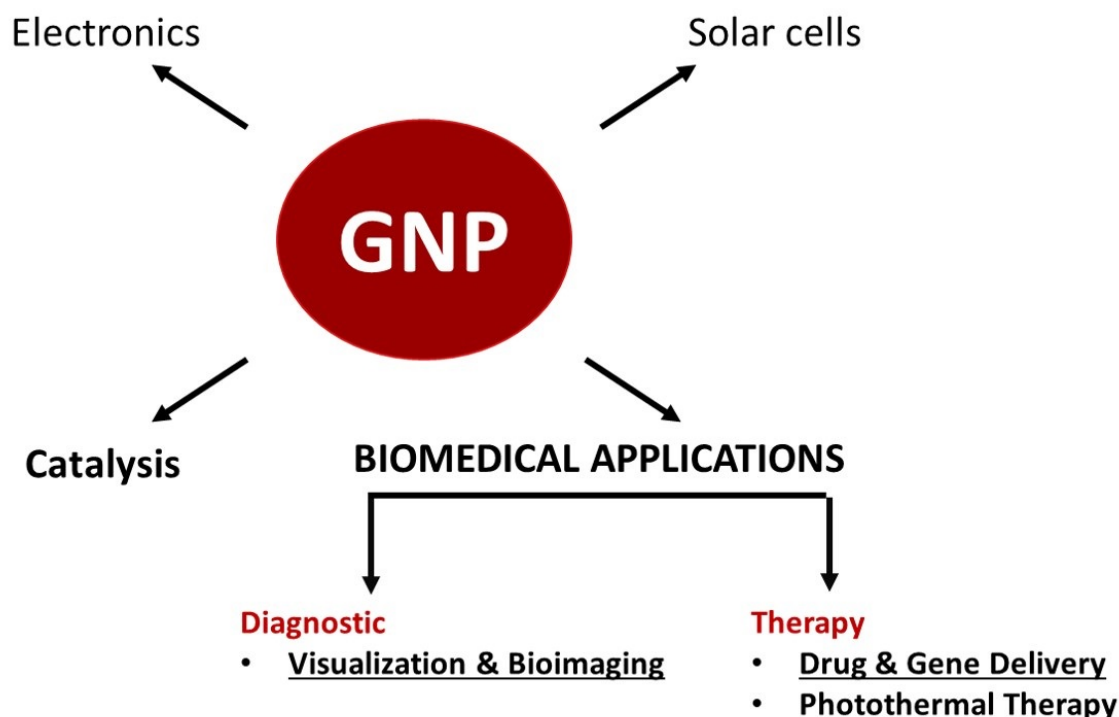


Figure 1.6: Illustration of application areas of GNPs.[7]

Moreover, they have a high X-ray absorption coefficient which makes them a great candidate as a contrast agent for computed tomography (CT). [66, 67, 68] For instance, X-ray absorption coefficient of iodine which is one of the widely used CT contrast agents is 1.94 at 100 keV where the X-ray absorption coefficient of GNPs is 5.16 at 100keV. Recently, GNPs have been used as photoacoustic (PA) contrast agents due to size and shape dependent, tunable absorption.[69] Among the other PA contrast agents such as organic dyes which suffer from photo-bleaching, GNPs preserve their photo stability, and this is extremely important in the clinic.

In addition to bioimaging applications, tremendous therapeutic applications of GNPs have been reported. Large surface/volume ratio, large number of surface functionality, controllable size and size distribution make them great candidates as nanocarriers. [17, 57, 70] GNPs can be modified with various biomolecules and ligands via thiol chemistry. [71, 72] The GNPs, produced with thiol chemistry, can be di-

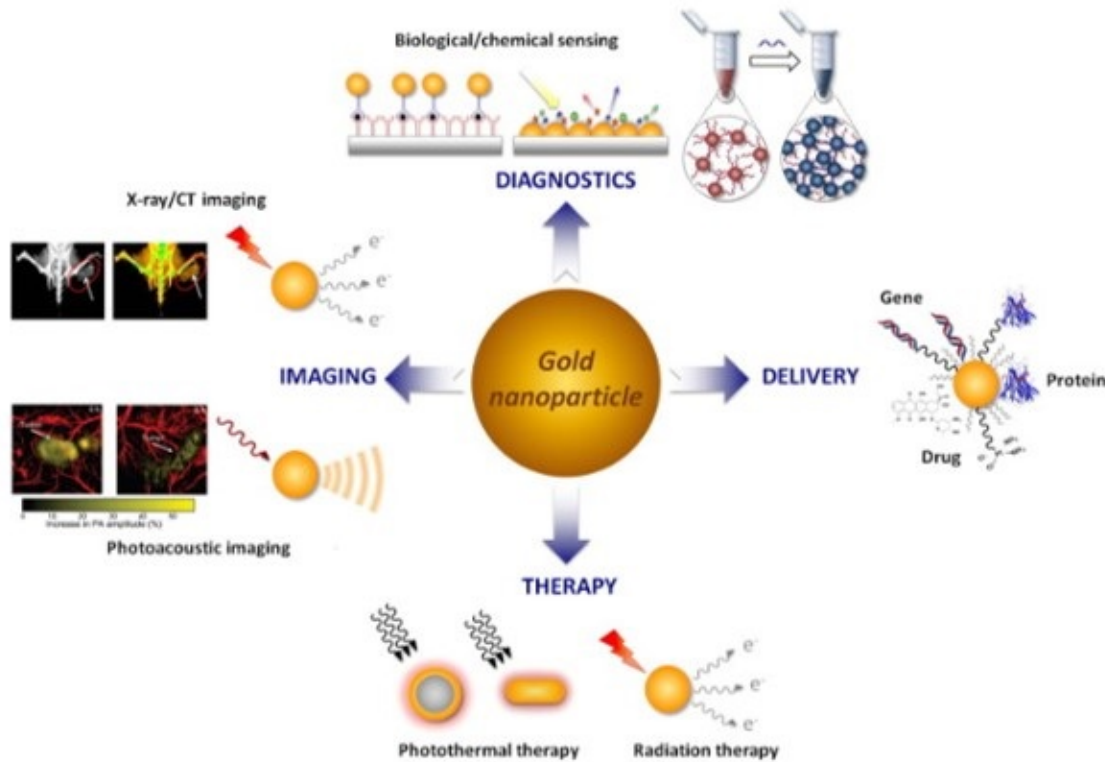


Figure 1.7: Illustration of biomedical application areas of GNPs.[7]

luted infinitely without losing stability.[72] Carrying drugs on NPs can increase the stability of the drug, solve solubility issues, enhance safety for healthy tissue, and/or improve cell internalization of the drug and may even provide a controlled release mechanism. [73] Drugs can be loaded on NP surfaces via electrostatic interactions or conjugated via simple conjugation chemistry such as amide, ester, ether, hydrazine, acetal bonds. Furthermore, GNPs can be decorated with specific molecules such as folic acid, RGD peptide or antibodies such as herceptin or cetuximab to target certain cell receptors to deliver the loaded drug to specific site which would improve the drug efficiency while reducing off-site effects. [74, 75, 76, 77] In case of electrostatic drug loading, the surface charge of the GNPs are designed as the opposite of the target drug. Cationic surface modification of GNPs enables them to carry therapeutic genes. The strong electrostatic interaction between cationic surface of GNPs and

anionic phosphate groups of genes protects them from enzymatic and nonenzymatic degradation during the delivery.[72, 78]

The tunable optical properties and the ability of converting absorbed light into heat via a series of non-radiative energy increased the popularity of GNPs in photothermal therapy (PTT). [79] The photothermal heating process of GNPs was observed by Link et al. by using femtosecond transient absorption spectroscopy.[80] As the laser pulses were absorbed by GNPs, free electrons in the plasmon bands were excited and created a pulse of hot electrons. The hot electron pulse transferred its heat to gold lattice via electron-phonon interactions and increased the temperature to thousands of degrees within ≈ 1 ps. Then the heat was transferred from the NP to its surrounding via phonon-phonon interactions within ≈ 100 ps increasing the temperature of the surrounding medium. As described earlier ability to create such an effect with excitation of the GNPs in NIR is needed for practical and more wider application of GNPs in PTT.

NIR-absorbing GNPs have great potential in PTT. Gold nanoshells with a size ca 110nm, gold nanorods with tunable size and LSPR band position are extensively studied for PTT. [81, 82] The difficulty in the reproducible synthesis and functionalization of those particles limit their therapeutic applications. GGS with absorbance in NIR posses great potential in PTT. One major limitation in GGS is the lack of stability beyond few days. Therefore, it is indeed critical to develop small, stable, NIR-absorbing GNPs with simple and reproducible synthetic methods for realization of practical PTT which may be easily combined with imaging techniques such as CT, photoacoustic imaging.

Catalytic Applications

Large surface to volume ratio, high molar absorption coefficient, LSPR band and chemical inertness make GNPs very attractive in catalysis. [83, 84, 85] GNPs have been utilized as catalyst in hydrogenation reactions of alpha beta-unsaturated aldehydes, cyclization reactions, C-C coupling reactions, oxidation reactions, etc. in the last

few decades. [58, 59, 86, 87, 88, 89] Recently, metal nanoparticles have been gaining attention for dye degradation due to large surface area to volume ratio.[86, 90] The main advantage of GNP based catalysts is preserving their catalytic activities under mild conditions.[91] The catalytic activity of GNPs is mainly related to their size, as the size of GNPs decreases, their catalytic activity increases.[92] Therefore, practical GNP synthesis, small, stable and functional GNPs are very valuable for industrial use of GNPs as catalysts.

1.2 Superparamagnetic Iron Oxides (SPIONs)

Iron oxide nanoparticles are the only FDA approved metal oxide nanoparticles and hence, subjected to many experimental and clinical studies. Superparamagnetic magnetite and maghemite are the most studied compositions due to biocompatibility, small sizes, and size-dependent unique and medically critical super-paramagnetic behavior. Indeed all materials may be considered magnetic since magnetism depends on the spin of electrons and their motion in the orbital, but they all differ in their response to an external magnetic field. Therefore, five different types of magnetism are described: diamagnetism, paramagnetism, ferromagnetism, antiferromagnetism, and ferrimagnetism.[8]

Diamagnetism is a very weak form of magnetism. Such materials have paired electronic structure and weakly magnetize in an external magnetic field in the opposite direction to the applied field. In the absence of the external field, no magnetic dipoles prevail. Paramagnetism is the alignment of atomic magnetic dipoles in the same direction with the external magnetic field, and magnetization is lost when the external field is removed. Paramagnetic materials have unpaired electrons, and associated magnetic dipoles/spins are randomly aligned, lacking cooperative interaction, hence are not magnetic in the absence of a magnetic field. On the other hand, in ferromagnetic materials, individual magnetic dipoles/spins are aligned in the same direction even in the absence of an external field. Therefore, ferromagnetism is also defined as permanent magnetism and is a strong magnetization. This ordering takes

place in close proximity in small volumes, and at large enough distances, other local domains may exist with another group of interacting spins. In each domain, magnetic dipoles are aligned in the same direction but not between different domains. As the magnetic field applied, magnetic dipoles in each domain begin to align in the same direction with the applied field. Therefore, domain boundaries disappear. When all dipoles are oriented with the applied field, magnetic saturation occurs. When the applied field is removed, domain walls appear again, but there will some magnetization left behind.[8] This phenomenon is called hysteresis is given in Figure 1.8. As the particle size increases, the hysteresis loop expands due to the slow-motion of dipoles in each domain. On the other hand, nanoparticles with sizes below 100 nm behave as an individual domain so that removal of the external field does not leave any residual magnetization behind, so no hysteresis is observed Figure 1.8. This phenomenon is called as super-paramagnetism, and because of this reason, superparamagnetism is a size-dependent phenomenon. [93, 94]

Iron, cobalt, and nickel are the most well-known ferromagnetic materials and magnetite and maghemite are possibly the most well-known superparamagnetic materials.

Antiferromagnetism and ferrimagnetism are observed especially in the oxide forms of transition metals due to antiparallel alignment of the spins between adjacent atoms or ions. Ferrimagnetic materials also have a net overall permanent magnetization, but antiferromagnetic materials have no net magnetic moment.[8]

Superparamagnetic iron oxides (SPIONs) are crystalline nanoparticles with crystal sizes between 10 to 100 nm and can be synthesized chemically in different ways such as co-precipitation, hydrothermal reactions, high-temperature decomposition and microemulsion [90] Co-precipitation method is the most common synthesis method to produce SPIONs which is also used in this thesis. This method was firstly reported by Massart.[95] Briefly in this method, ferric (Fe^{+3}) and ferrous (Fe^{+2}) ions (molar ratio 1:2) are mixed in basic aqueous solution at a desired temperature under an inert atmosphere. Even though the resulting particles are polydisperse, the scale of the synthesis can be adjusted from milligrams to kilograms. [96] Additionally, particle

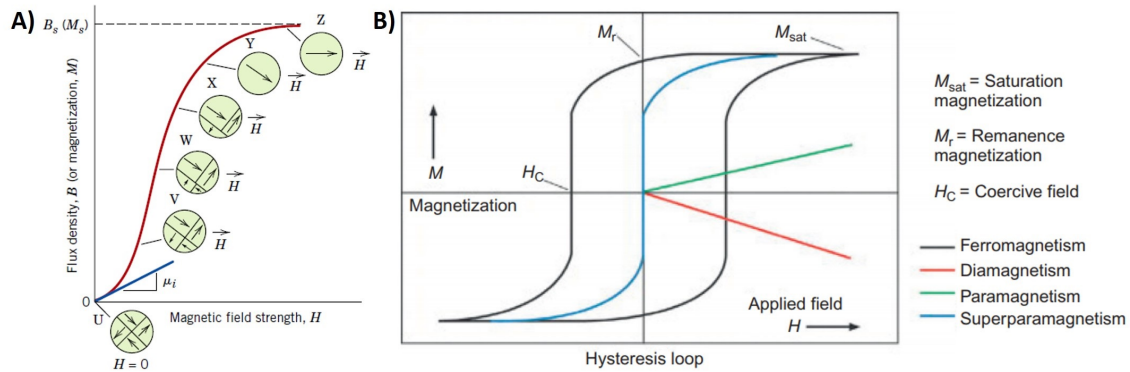


Figure 1.8: Illustration of (A) magnetization behaviour of ferro and ferri magnetic materials.[8] (B) hysteresis loops of ferro- and superparamagnetic materials with comparison of para- and diamagnetic behaviours.[9]

size can be tuned from 2 to 20 nm by changing the reaction temperature, pH and the reactant concentrations. The original synthesis produces bare Fe_3O_4 , which precipitates out of the solution. . To increase the stability, prevent oxidation, increase the solubility, and provide functional groups for stability and to be used in further modifications, SPIONs need to be coated with functional molecules. [97, 98] The coating material can be added during the synthesis or as a post-synthesis procedure; however, later provides larger aggregates. In-sit coating of the growing crystal provides smaller particles with more uniform size distribution and much less aggregation. [99]

1.2.1 Applications of SPIONs

SPIONs attracts researchers due to their strong magnetization, high surface area to volume ratios, low toxicity, chemical inertness, an easy and large scale synthesis, coating and functionalization possibilities.[100] SPIONs have been used in variety of applications such as biomedical applications, catalysis, waste water treatment, magnetic storage media and energy applications (Figure 1.9).[10]

SPIONs are under heavy investigation in both diagnostic and therapeutic applications (Figure 1.10). The well known diagnostic application of SPIONs is to be used as a magnetic contrast agent in magnetic resonance imaging (MRI).

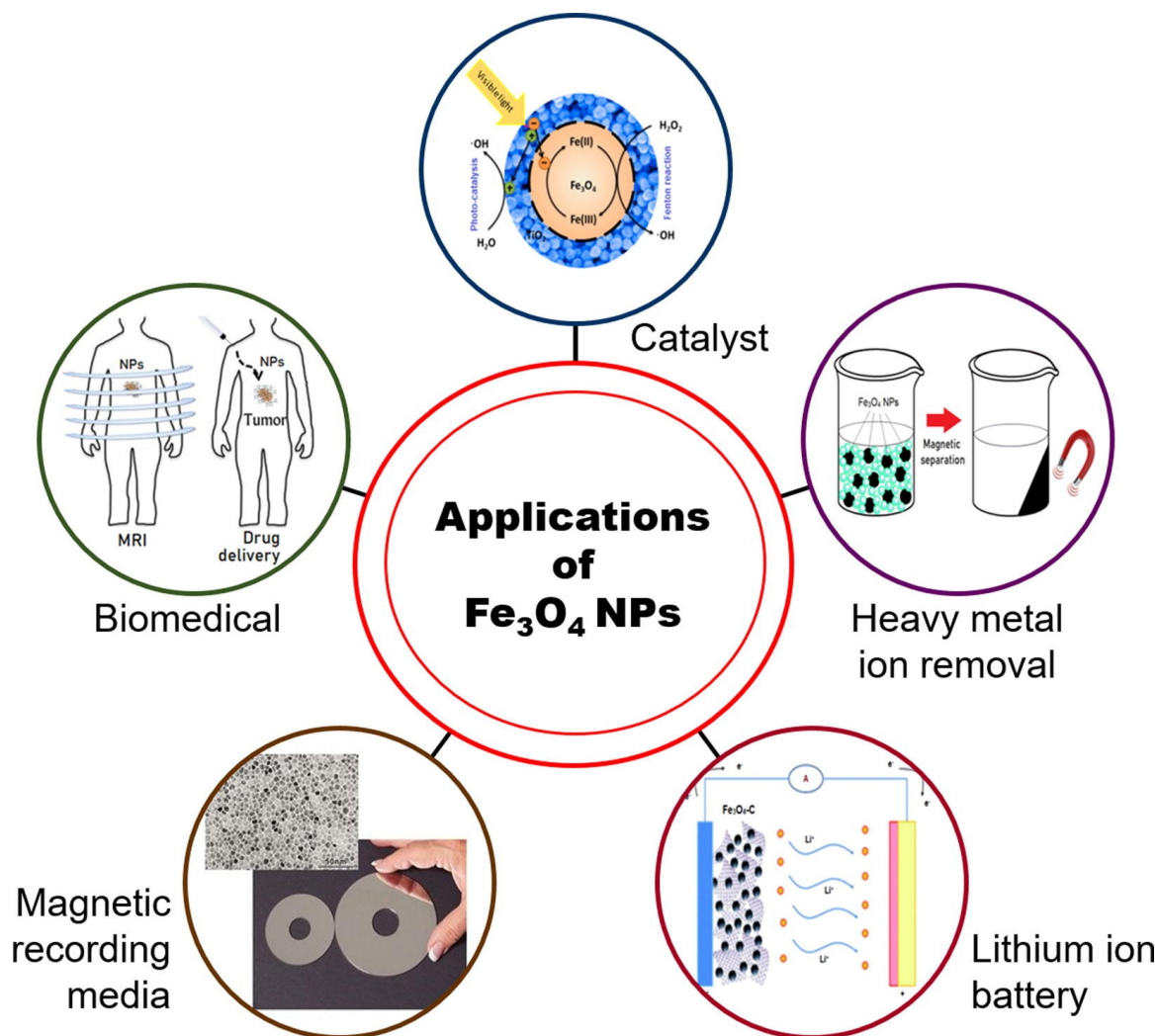


Figure 1.9: Illustration of application areas of SPIONs.[10]

Feridex[®] and Resovist[®] are the two Food and Drug Administration (FDA) approved SPION based contrast agents for the organs of reticuloendothelial system (RES).[101] The former one is coated with dextran with hydrodynamic size 120-180nm, the latter one is coated with carboxydextran with hydrodynamic size 45-60nm. Modified SPIONs with quantum dots and dyes are also used as a probe in Positron emission tomography (PET), near-infrared fluorescence (NIRF) imaging and in biosensors for detection of biomolecules.[102]

In addition to diagnostic applications, SPIONs are well adopted as therapeutics



Figure 1.10: Illustration of biomedical application areas of SPIONs.[11]

due to low toxicity and the controllable movement in the blood circulation with an external field.[103, 104] SPIONs can be used as a nanocarrier for anionic or cationic drug and gene delivery depending on its surface charge. Moreover, SPIONs may act as therapeutic agents creating magnetic hyperthermia and very recently by photothermal therapy. [104] In magnetic hyperthermia, the heat is generated with an applied magnetic field. The hysteresis loss and relaxational loss produces the heat which depends on the particle size. Hysteresis loss occurs in large particles (over 100 nm) due to multiple magnetic domains. As the particle size decreases, the number of magnetic

domains decreases as explained in the super-paramagnetism. Therefore, heat is produced by Neel relaxation and Brownian relaxation when the particle is exposed to an applied magnetic field due to the realignment of the magnetic moment. [105] Photothermal therapy is quite new for SPIONs, and the origin is still under investigation. Unlike GNPs, SPIONs developed for drug delivery and or for imaging may be utilized directly for PTT in the NIR region. Moreover, heating capacity of SPIONs is not wavelength-dependent within a broad range in the NIR. The information was proved with a study done by our group.[106] They have completed sets of controlled experiments with irradiating PAA coated SPIONs with a green-pumped continuous-wave Ti^{+3} :sapphire laser in the range of 728-838 nm and observed the temperature change was not dependent on the excitation wavelength, sample attenuation or intensity of the laser irradiation but on the laser power.

SPIONs have been utilized also as an eco-friendly hetero-catalyst in different applications, due to easy recovery in the presence of external magnetic fields for reuse and adjustable surface functionalization. [99] In addition, easy large scale and low-cost synthesis and strong adsorption capacity of SPIONs are quite desired properties for catalysts.[107, 108] SPIONs have been utilized as a catalyst in organic chemistry reactions, photocatalysis, and nanoasorbent for wastewater treatments in the literature.[109, 100, 110, 108]

1.3 The aim and proposal of this thesis

Nanoparticle term is defined as a material that has at least one dimension with a size less than 100 nm according to ISO and ASTM standards.[111] Research on nanoparticles and their applications was accelerated tremendously over the past few decades. Over four thousand research articles on metal nanoparticles were published on Web of Science in 2018 so far. Main problems, especially in gold nanoparticle literature, are lack of the reproducibility of reported methods, poor control in size and shape, and lack of stability of GNPs. Based on such problems in the gold literature, one aim of this thesis is the development of simple, relatively economical and reproducible

procedures for the synthesis of gold nanoparticles which are functional and highly stable over the years which is quite rare in the literature. The second is to produce a broad portfolio of functional gold nanoparticles, cationic and anionic, by using different reducing and coating materials for the delivery of anionic and cationic cargo in medicine. Specifically, gene delivery and drug delivery in cancer treatment, IgG delivery across BBB in the treatment of sepsis and catalysis of azo dye degradation were studied with such functional, small, and stable GNPs. The third goal of this thesis work is to develop stable GNPs with absorbance in the near-infrared (NIR) region to be utilized in photothermal therapy. The last goal of this thesis work is to develop a one-pot green synthesis of a sulfonic acid functional superparamagnetic iron oxide for medical purposes, with potential for targeting infections and potential in photothermal therapies due to absorbance in the NIR, which is highly desired in the field of phototherapies.

In the second and third chapter, small, colloiddally stable, reproducible, aqueous, single-step synthesis of cationic gold nanoparticles were developed by using branched and linear polyethyleneimine (PEI). PEI is a popular amine-rich, cationic polyelectrolyte, known as the best non-viral gene transfection agent in the field of medicine. It is often combined with nanoparticles for stability, size control, targeting, imaging and/or combination therapy of inorganic nanoparticles. Almost always this is achieved via conjugation or electrostatic adsorption of PEI on pre-formed nanoparticles. A combination of GNPs with PEI would bring together many advantages. Initial ideas include gene transfection, catalysis, drug delivery, phototherapies, and medical imaging. One interesting approach in the synthesis of PEI bound GNPs is a direct reduction of auric acid by PEI. Such a simple approach is both scientifically interesting and economically and practically attractive for industrial adaptation of the procedure. The scientific challenge is indeed expressed by the reports which indicated the failure of such approach in non-reducing solvents, with linear PEI and low molecular weight polymers. The hypothesis of this thesis is the pH dependence of reducing power and stabilizing the effect of PEI. Since the protonation of PEI will

depend on the pH of the environment, it should be possible to find an appropriate pH that would provide reduction and stability at the same time. Therefore, in chapters 2 and 3, reaction parameters including pH of the reaction, polymer molecular weight and topology, polymer/Au ratio and reaction time were investigated in successful synthesis of small and colloidal stable PEI coated GNPs from the aqueous solutions of auric acid. bPEI easily dissolves in water, but the dissolution of lPEI requires either protonation or high temperatures; hence, this is also considered as a variable for both reduction, surface adsorption, and stability of GNPs. Post-synthetic cleaning procedures and storage pH are indeed important to maintain the desired properties of GNPs, which is usually not detailed in the literature, which is part of the reasons for the irreproducibility of methods reported in the literature. Since our group works on PEI based gene transfection for many years, investigation of the biocompatibility of bPEI coated GNPs and their use in gene transfection to cancer cells are also included in this thesis work (Chapter 2). GNPs are widely used for catalysis, and one such application is the degradation of toxic azo-dyes. Such dyes are either anionic, cationic, or zwitterionic. Hence, GNPs with the opposite charge to target dye are designed and tested in the literature. It would be essential to develop a single GNP catalyst that can bind and catalyze the degradation of both cationic and anionic dyes. Considering the pH-dependent protonation of PEI, its solubility in aqueous and organic solvents, one of the hypotheses of this thesis is the potential of GNP-PEI as a universal catalyst for a variety of azo-dyes at appropriate pH values. In Chapter 3, this potential is thoroughly investigated as a function of pH and polymer MW using cationic methylene blue (MB) and anionic methylene orange (MO) as conventional model dyes.

Ability to produce stable, small, aqueous colloidal GNPs provided encouragement to develop small GNPs that can pass BBB, which is very important, especially in the treatment of "sepsis". One of the most promising therapeutic agents in sepsis is IgG, but it does not pass BBB in therapeutic dose. One of the main goals of this thesis is to develop small, stable GNPs to deliver IgG to the brain in ther-

apeutic doses, specifically achieve more than 0.1 % delivery across BBB. For this purpose, 3-mercaptopropionic acid (3MPA) coated GNPs with the surface COOH groups that can be used for conjugation of IgG and glucose for selective passage across BBB is suggested. One phase Brust-Schiffrin method was modified to synthesize 3-mercaptopropionic acid (3MPA) coated GNPs in water. For the conjugation of IgG, acid cleavable hydrazone linkage and for glucose, a PEG spacer enhancing the availability of glucose is suggested. Parameters of synthetic protocols, cleaning procedures, sterilization and storage conditions need to be carefully investigated since IgG is a big molecule and prone for bacteria growth and polyclonal IgG has a wide range of isoelectric points. These nanoparticles are designed for a joint project co-directed by Prof. Mehmet Kaya at KUH for treatment for sepsis.

Metal nanoparticles, metal oxides, and sulfides are being investigated as photosensitizers for catalytic reactions and for photothermal therapy of cancer and biofilms. One of the major cavity in the field is to achieve enhanced activity with an easy to make, biocompatible, stable nanoparticles, and the ability to sensitize nanoparticles with NIR lasers. GNPs are also attractive in this field since excitation at the surface plasmon peak wavelength provides successful examples. Gold nanorods are promising, but their synthesis is not that practical or reproducible. One interesting composition emerged as gold-gold sulfide (GGS) nanoparticles, but there is a debate on their nature in the literature, and existing examples lack suitable coatings and hence do not have colloidal stability. In chapter 5, techniques developed in this thesis work are suggested as methods to produce NIR absorbing cationic (bPEI), anionic (3MPA) and drug (vincristine) coated NIR absorbing GGS nanoparticle with excellent colloidal stability. These particles are designed with a motivation of achieving combined/targeted therapies such as chemo-photothermal therapy and photodynamic-photothermal therapies by loading drugs/targeting molecules or photosensitizers to these GGS nanoparticles.

In the last chapter, an extension of our efforts started with GNPs in phototherapies, which directed part of the thesis work to NIR sensitive SPIONs will be dis-

cussed. Throughout this thesis work, studies focused on directing nanoparticles to the site of interest, therapeutic nanoparticles in various diseases were actually followed carefully, and many exploratory studies were conducted (not reported here). Sulfonic acid functional polymers and drugs seem to have a special affinity to infection sites, especially RA. This stimulated the last motivation of this thesis: development of sulfonated nanoparticles for natural infection/RA targeting, and image combined therapies. Vincristine coated GGS is indeed one composition suggested for such a target. Another composition suggested here is sulfonic acid functionalized SPIONs with MRI imaging, magnetic dragging, magnetic hyperthermia, and photothermal therapy potential that are being investigated in our group. A novel, one-pot aqueous synthesis method utilizing sulfonic acid functional silane as a coating is suggested for the synthesis. This is to provide a greener synthetic route compared to similar compositions present in the literature.

Chapter 2

CATIONIC GOLD NANOPARTICLES SYNTHESIZED WITH USING BRANCHED PEI

2.1 pH and molecular weight dependence of auric acid reduction by branched polyethylenimine and the gene transfection efficiency of the cationic gold nanoparticles thereof

Gold nanoparticles (GNPs) have been around for a very long time, and are still highly attractive due to unique size and shape dependent optical and electronic properties, especially in catalysis, sensors, conducting materials, etc.[24, 59, 112, 113, 114] In medicine, GNPs hold a great potential due to inert and nontoxic nature added to diagnostic and therapeutic potential.[44, 61, 115] They are under heavy investigation in drug and gene delivery, photothermal therapy, optical imaging and computed tomography (CT).[59, 116, 117, 118, 119] In colloidal form, GNPs need to be stabilized with an organic material which is usually a small bifunctional molecule or a polyelectrolyte.[117, 120] Utilization of such during the synthesis is a valuable tool to control the particle size and shape, as well. Two common methods to synthesize GNPs are the Turkevich and Brust-Schiffrin method.[27, 34] The former produces large hydrophilic particles (8-120 nm) in aqueous medium, the latter produces smaller but hydrophobic particles (1-3 nm). One attractive composition would be GNPs coated with polyethylenimine (PEI), which is the golden standard for non-viral gene delivery.[121] This is usually done in a post-synthetic step in which PEI is conjugated to the initial surface coating of GNPs.[121] An alternative, simple and cheaper way is the direct reduction of auric acid with PEI which simultaneously forms a cationic PEI coating on GNPs as first reported by Sun.[122, 123] This initial report

showed that gold nanoparticles or plates can be produced this way with linear PEI (423 Da) depending on the procedure. There are few more studies reporting direct reduction of auric acid with PEI producing GNPs in a crystal size regime of 6-17 nm utilizing different molecular weight, morphology (linear or branched) reaction temperature, UV-irradiation and solvents.[117, 124, 125, 126, 127, 128, 129] Since each report used a different combination of these variables, it is difficult to decouple the effect of each variable on particle properties. Sun reported increasing particle size with increasing PEI/Au ratio using 25 kDa bPEI.[130] Kretschmer reported Au clusters with tunable overall size between 30-200 nm using 25 kDa bPEI in DMF at 150°C and poor stability with 0.6 and 1.8 kDa bPEI.[125] Since DMF has a reducing power as well, reaction was repeated in water but results were unsatisfactory. Interestingly, influence of the reaction pH, hence protonation of bPEI, has not been studied yet as an important variable. Since the lone pair electrons of the PEI is held responsible from both reduction and surface adsorption, pH should be one of the most important reaction variables in controlling the rate of reduction, size and stability of the resulting GNPs. To the best of our knowledge, there is just one report which studied the effect of acidification by either introducing CO₂ to the reaction which causes the thermally reversible carbamate formation, or used fixed amount of HCl with varying amounts of bPEI to alter the pH.[127] Hence, it is difficult to decouple the effect of pH on the process and properties of GNPs from the effects of bPEI/Au concentration or from the presence of carbamates. Yet, they suggest that increasing pH from 3.0 to 9.0, increases the hydrodynamic size and clustering as well as a red shift and broadening in the plasmon resonance band of GNPs. Here, we study the direct reduction of auric acid directly in water, which is a non-reducing and biologically acceptable solvent, with bPEI, as a simple, economic and safe method to develop small cationic GNPs which would be especially valuable for biomedical applications. We primarily investigated the influence of the reaction pH and molecular weight of bPEI at constant bPEI/Au ratio and reaction time as well as bPEI/Au weight ratio at fixed molecular weight, pH and reaction time on the properties of produced cationic GNPs,

specifically on particle size, stability, charge and shape.

2.2 Experimental

2.2.1 Materials

Gold (III) chloride solution 30 wt % in dilute HCl (HAuCl_4) was purchased from Aldrich (Germany). 0.6, 1.8, 10 and 25 kDa branched polyethylenimine (PEI) were purchased from Aldrich (Germany). Centrifugal filters with 3, 10 and 30 kDa cut-off membranes were purchased from Sartorius. Dulbecco's modified Eagles medium (DMEM, with 4.5 g/L glucose, with l-glutamine, sodium pyruvate and phenol red), penicillin/streptomycin and trypsin-EDTA were purchased from Multicell, Wisent Inc. (Canada). Fetal bovine serum (FBS) was purchased from Capricorn Scientific GmbH (Germany). Opti-MEM I reduced serum medium was obtained from Gibco (Invitrogen, USA). Phosphate buffered saline (PBS) tablets and thiazolyl blue tetrazolium bromide (MTT) were purchased from Biomatik Corp. (Canada). Dimethyl sulfoxide Hybri- Max and 4,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma (USA). Paraformaldehyde solution (4 %) in PBS was purchased from Santa Cruz Biotechnology, Inc. (USA). All of the 6, 12 and 96-well plates were supplied by Nest Biotechnology Co. Ltd. (China). Human embryonic kidney (HEK 293T) cell line was a kind gift of Dr. Nathan Lack (School of Medicine, Koc University, Istanbul, Turkey).

2.2.2 Instrumentation

UV-vis absorption spectra were recorded on a Shimadzu UV-3600 spectrophotometer. Transmission electron microscopy (TEM) images were taken on a Techani G2 F30 brightfield high resolution (HR) TEM (acceleration voltage = 200 kV) using samples deposited on carbon coated Cu-grid from dilute solutions at UNAM-Ankara. Dynamic light scattering (DLS) and zeta potential measurements were done using a Malvern Zetasizer Nano-ZS. For XPS and XRD analysis, PEI-Au solutions were freeze-dried

to obtain samples in the powder form. XPS analysis were performed on a Thermo Scientific K-Alpha XPS with Al K monochromatic radiation (1486.3 eV). Powdered samples were put on a carbon adhesive tape. 50.0 eV pass energy was used for the scans with a flood gun with a 400 mm spot size. A base pressure lower than 910^{-9} mbar and experimental pressure of 110^{-7} mbar were achieved. The C1s peak at 284.5 eV was used as a reference. For XRD measurements, powdered samples were placed on a silicon holder and analysed with a D8 Advance Bruker instrument with Cu K α radiation ($= 1.5406 \text{ \AA}$) between 2θ angles of 10° and 80° .

2.2.3 Synthesis of bPEI coated GNPs

GNPs were synthesized by the reduction of H AuCl_4 with branched PEI as the only capping and reducing agent. 6.810^{-5} M H AuCl_4 solution was mixed with 200 μl aqueous bPEI (10 mg/ml) solution for each bPEI molecular weight. Reactions were performed in 20 ml glass vial with a teflon cap. Reaction mixtures were placed in a preheated (95°C) silicon oil and stirred at constant speed (500 rpm) for about 10-40 min. Time is specified for each reaction in tables and figures. The change in the colour of reaction mixture from yellow to reddish indicates reduction of gold from Au^{+3} to Au^{+1} to Au^0 . After cooling down the solution to room temperature, bPEI-Au nanoparticles were washed with de-ionized water couple of times using centrifugal filters with appropriate molecular weight cut-off membrane. Resulting GNP dispersions were stored in a vial covered with an aluminium foil and kept in 4° . In order to investigate the effect of pH on GNPs, separate experiments were performed. Before placing the reaction mixture into the preheated oil bath, its pH was adjusted with 0.5M sodium hydroxide or acetic acid solution and was mixed for 2 minutes at room temperature.

2.2.4 Cell Culture

Human embryonic kidney (HEK293T) cells were cultured in DMEM complete medium supplemented with 10 % (v/v) FBS and 1 % (v/v) antibiotics (penicillin/streptomycin)

in 5 % CO₂-humidified incubator at 37°C.

2.2.5 Determination of Cell Viability

Cells were plated at a density of 125000 cells/ml in regular growth medium consisting of DMEM supplemented with 10% FBS and 1% penicillin-streptomycin in 96-well plates in a total volume of 200 μ l. After 24 hours cells reached 80 - 90% confluency and the medium was replaced with 150 μ l fresh medium and 50 μ l sterile water containing the Au-PEI nanoparticles (sterilized by filtering through 0.2 micron sterile syringe filter) to provide the indicated concentration of nanoparticles in Figure 2.6. After 24 hours of incubation with nanoparticles, standard MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay was performed by washing the cells and adding 150 μ l of fresh growth medium and 50 μ l 5 mg/ml MTT solution into each well. After incubating the cells for 4 hours, the MTT solution was aspirated and 200 μ l of a 1:1 ethanol: DMSO solution was added to solubilize the dye. Elisa Plate Reader was used to carry out and quantify the dye concentration at 600 and 630 nm. All data represents the average of 2-4 replica. All viability data is reported as normalized with respect to non-treated control cells.

2.2.6 Qualitative evaluation of transfection

HEK 293T cells at a density of 105 cells/well were seeded 24 h prior to transfection into 12-well plates in 2 mL of DMEM complete medium (supplemented with 10 % FBS, 1 % penicillin-streptomycin). Different amounts of GNPs were mixed with 2.33 μ g/mL of pMax-GFP vector DNA (Lonza) in Opti-MEM I reduced serum medium, separately and incubated for 20 min at room temperature (RT). Then, these complexes were added to cells at 60-70 % confluency and incubated 48 h post-transfection. FuGENE HD transfection reagent (Promega, USA) was used as a positive control. Reagent to pDNA ratio of 3:1 (μ L/ μ g) was used as recommended by the producer. Untreated cells were used as a negative control. For the microscopy study, cell nuclei were stained with DAPI at a final concentration of 1 μ g/mL. Cells were allowed to incubate for

20 min at RT and washed with PBS. After staining procedure, cells were fixed in 4 % paraformaldehyde and incubated for another 20 min at RT and then, washed with PBS. Finally, 2 mL of PBS was left in each well to prevent the cells from air-drying. Images were taken on an inverted fluorescence microscope (Olympus-Xcellence Real-Time Life Science Microscopy) and processed with ImageJ analysis program.[131]

2.2.7 Quantitative determination of transfection efficiency

Quantitative determination of transfection efficiency was achieved by flow cytometry at optimal ratio of GNP/pDNA (8 $\mu\text{g}/\text{mL}$ GNPs/2.33 $\mu\text{g}/\text{ml}$ pDNA) HEK 293T cells were seeded into 6-well plates at a concentration of 2×10^5 cells/well and incubated for 24 h in 5 % CO⁺² and at 37 °C. Next day, GNP/pMax-GFP complexes were introduced to growing cells with fresh culture medium and the cells were incubated for another 48 h. Then, the transfected cells were detached with trypsin-EDTA from wells after being washed with PBS, and were re-suspended in 500 mL PBS containing 1 % FBS. Finally, transfection efficiency was assessed as the percentage of cells expressing green fluorescent protein using BD Accuri C6 flow cytometer with the C6 Software (BD Biosciences, San Jose, CA, USA). FuGENE HD transfection reagent, untreated cells and naked pDNA treated cells were also performed with the same procedure. All experiments were repeated three times.

2.3 Results and Discussion

In a typical reaction procedure, aqueous auric acid solution and bPEI solution are mixed at room temperature (RT) at bPEI/Au weight ratio of 1:7, and then placed into an oil bath at 95°C. Initially, bPEI 25kDa was used since it is the most widely used one in biomedical applications. The bPEI-auric acid mixture has a characteristic yellow colour due to Au⁺³ which turns slowly into red when placed into the oil bath and eventually into purple at later stages (ca 30 min). Electron transfer from amine groups of bPEI to Au⁺³, reduces Au⁺³, and creates metallic gold. This transformation was also followed with UV-vis spectroscopy (Figure 2.1a). Initial bPEI/Au⁺³ solution

has an absorbance at 360 nm. As the reduction takes place, the characteristic surface plasmon band (SPB) of GNPs was observed at around 520 nm. At longer reaction times, peak maxima shifted to 530-540 nm due to particle growth. Under these conditions, pH of the initial bPEI/Au mixture was 7.6. In a series of reactions, keeping everything else the same, pH of the initial mixture was set to 3.6, 5.0, 7.0 and 9.0 with acetic acid and sodium hydroxide. Absorption spectra of the GNPs synthesized at these pH values in 20 min are shown in Figure 2.1b and compared with the one prepared without pH adjustment (control: pH 7.6).

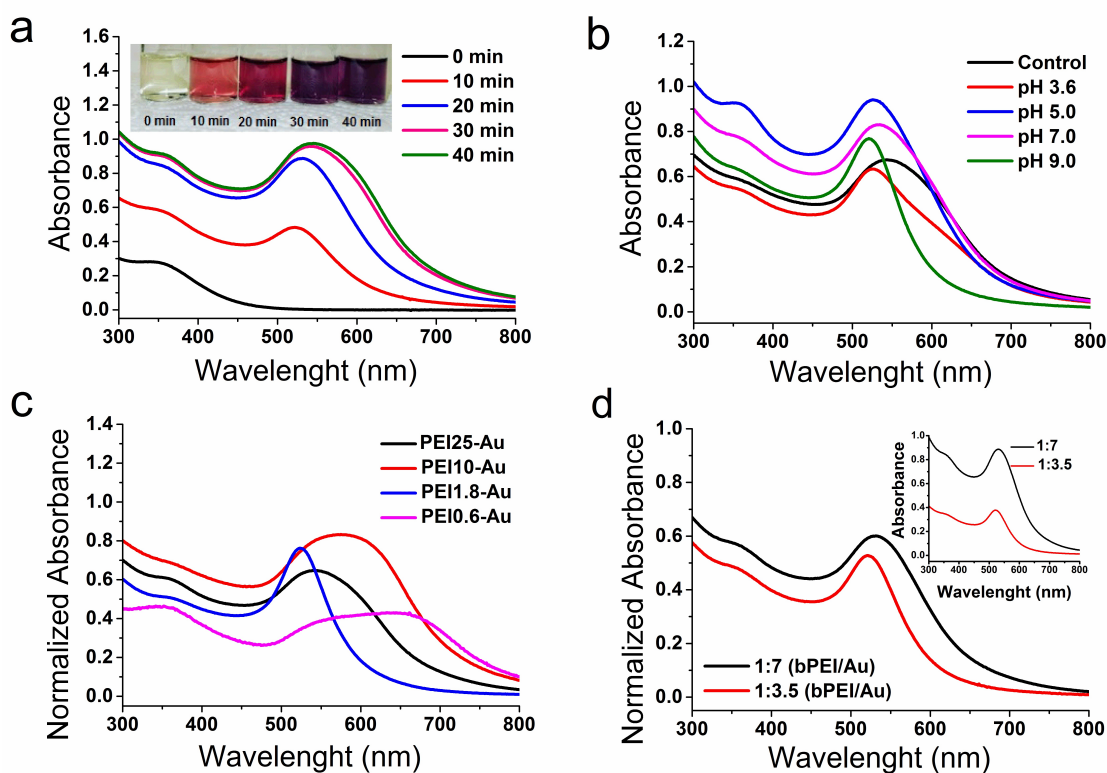


Figure 2.1: Absorbance spectra of washed GNPs synthesized at a) different reaction times, b) different pH conditions (control: pH 7.6), c) different molecular weight of PEI, d) different concentration of HAuCl₄. Inset: Un-normalized absorbance spectra. 25kDa PEI and bPEI/Au weight ratio of 1:7 were used for synthesis of GNPs shown in a, b and c. Reaction time was fixed to 20 min in b, c and d. Reaction pH was 7.6 in a, c and d.

The surface plasmon peak of GNPs prepared at pH 9.0 with a sharp absorbance maxima at 520 nm is significantly different than the others. At basic pH, protonation of bPEI is the least, hence it has maximum number of free amines to donate electrons for the reduction and for the surface adsorption which would restrict particle growth and provide stabilization. Hydrodynamic size of these GNPs are 6.7 nm with a surface charge around +33 mV (Table 2.1). On the other extreme, GNPs synthesized at pH 3.6 have a broad SPB indicating formation of particles in broad size distribution and possibly with some aggregation which is supported by a much larger hydrodynamic size (43 nm) (Table 2.1 1). Such a low pH is suitable for protonation of all three types of amine groups available in bPEI. This renders particles highly cationic with the zeta potential of +61.6 mV but on the other hand reduces the amount of free lone pair electrons necessary for reduction and surface adsorption (Table 2.1 1). Besides, electrostatic repulsion of the repeat units may hinder effective surface adsorption. GNPs synthesized at pH 5.0, 7.0 and 7.6 (no pH adjustment) have similar absorbance profile with ca 20-37 nm hydrodynamic size and +49-53 mV zeta potential. GNP synthesized at pH 9.0 kept its colloidal stability over 1.5 year at the moment during RT storage, but GNPs synthesized at pH 7.0 and 5.0 were precipitated after 1 year and those which were prepared at pH 3.6 and control kept colloidal stability but aggregated as indicated with a colour change into purple (Figure 2.2). Enhanced stability of GNPs synthesized at basic pH is probably due to better surface binding. Actually, production of sharper absorbance peak and smaller particles at basic pH is contradictory to the results reported previously which suggested nanoparticle clustering at basic pH.²³ Yet, such aggregation is possibly due to significantly higher PEI concentration used in that study in order to achieve a basic pH.

When XPS spectra of the PEI25-Au synthesized at pH 3.0 and 9.0 were compared, clear differences were observed (Figure 2.3). There are two different types of N 1s in both samples with binding energy (BE) at ca 399 and 400 eV corresponding to the free and bound or oxidized amines, respectively. However, the amount of the N 1s with the higher BE is about 64% and 9% of all amines for the GNPs synthesized at

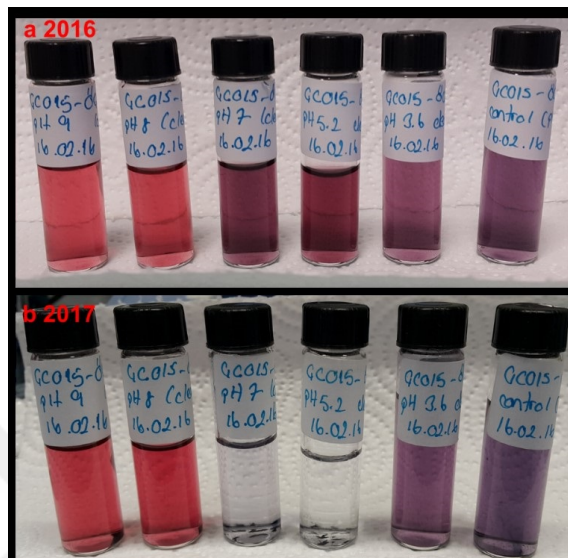


Figure 2.2: Photos of PEI25-Au synthesized at pH 9, 8, 7, 5.2, 3.6 and control (no pH adjustment) from left to right a) after being washed following the synthesis step and b) after 1 year storage at room temperature.

basic and acidic pH, respectively. Corresponding differences exist in the C 1s and Au 4f, as well. There are three types of carbons with C 1s BE at ca 284.5, 285 and 287 eV. Two of the higher BE ones correspond to C-N and C=N (Table 2.2). Formation of the latter was also suggested by Kretschmer during the high temperature gold reduction with PEI.²¹ XPS indicates more than two fold increase in the ratio of C=N/C-N if the synthesis was performed at basic pH rather than the acidic (Table 2.3). Analysis of the Au 4f indicates the presence of Au⁰ and Au⁺¹ in both cases, but with more reduced gold at the basic pH. When N/Au ratio was compared, there seems to be more N bound to Au at the basic pH. All of these data suggests that at basic pH since lone pair electrons of the amine groups are available, there is more gold reduction, some amine oxidation and more amine binding to GNP surface which agrees with smaller size and better stability for the GNPs prepared at basic pH.

Molecular weight of the polymeric coating is important for the particle size and the stability. Hence, the influence of molecular weight on properties of GNPs were investigated using 0.6, 1.8 and 10 kDa bPEI, as well (Figure 2.4). bPEI 0.6kDa pro-

Table 2.1: Influence of pH, MW and mass ratio on the properties of GNPs

Sample name	PEI MW (kDa)	PEI:Au (weight ratio)	Size ^a (nm)	PDI ^b	Zeta Potential (mV)
Control	25	1:7	36.60	0.761	+52.1
pH 3.6	25	1:7	42.91	0.272	+61.6
pH 5	25	1:7	25.80	0.255	+53.1
pH 7	25	1:7	21.04	0.228	+49.5
pH 9	25	1:7	6.70	0.546	+33.7
PEI0.6-Au	0.6	1:7	109.10	0.410	+32.8
PEI1.8-Au	1.8	1:7	7.69	0.444	+37.1
PEI10-Au	10	1:7	50.45	0.293	+48.6
PEI25-Au	25	1:3.5	43.80	0.407	+40.9

^a Number average hydrodynamic size and ^b polydispersity index (PDI) calculated from DLS measurement

Table 2.2: Binding energies (eV) of N1s, C1s and Au 4f core level in PEI25-Au synthesized at pH 9.5 and 3.5.

	N1s	N1s	C1s	C1s	Au4f	Au4f
	Free amines	Reduced amines	C-N	C=N	Au ⁺³	Au ⁺
at pH 9.5	399.09	400.23	284.86	287.21	83.84	84.86
at pH 3.5	399.02	400.63	285.32	287.88	83.06	84.08

duced more aggregated colloidal particles (PEI0.6- Au) as indicated with the purple colour of the solution (Figure 2.1c, Figure 2.4). Despite the strongly positive surface charge, short chain polymers may not provide enough steric stabilization. Poor stabilization causes aggregation and large size distribution (109 nm, Table 2.1). bPEI 10kDa formed smaller particles than bPEI 0.6kDa but larger than bPEI 25kDa with

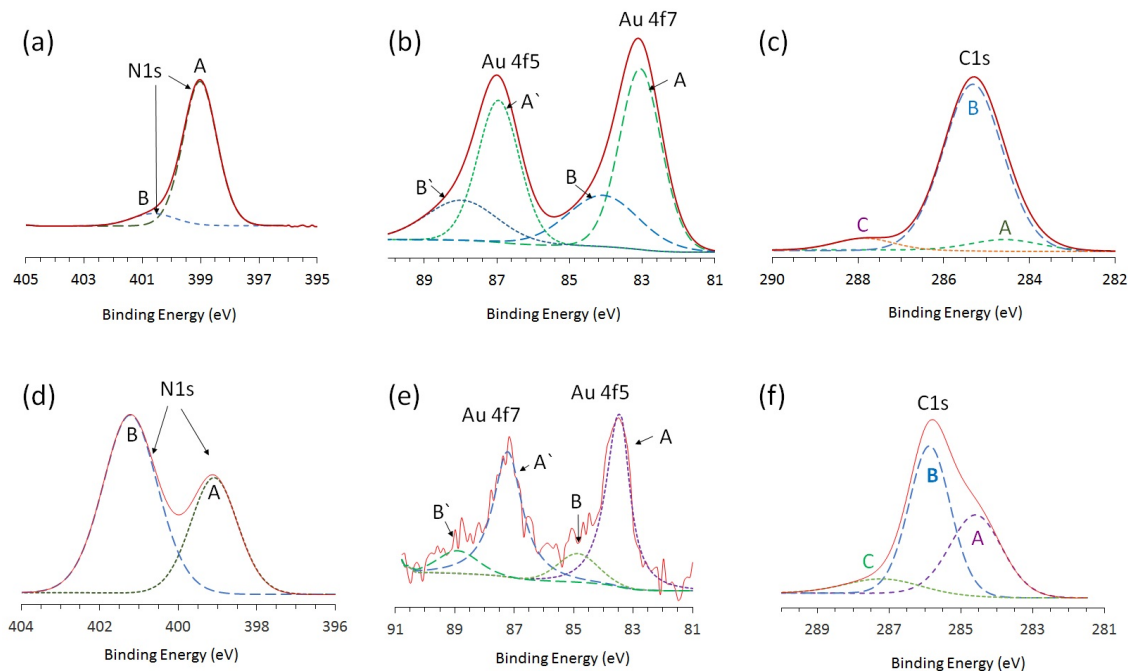


Figure 2.3: XPS spectra of PEI25-Au synthesized at pH 3.6 (a,b,c) and at pH 9.5 (d,e,f).

Table 2.3: Atomic ratio of C, N, Au in the PEI25-Au synthesized at different pH values

Atomic ratio	pH 9.5	pH 3.5
N_{bound}/N_{tot}	0.64	0.09
$C=N/C_{tot}$	0.15	0.065
Au^0/Au_{tot}	0.80	0.71
N/Au	428	291
N_{400}/Au_{83}	342.5	40.60

50 nm hydrodynamic size and +48.6 mV zeta potential. Its absorbance peak was still very broad (Table 2.1), Figure 2.1c, and Figure 2.4). Surprisingly, GNPs produced with bPEI 1.8kDa (PEI1.8-Au) have the sharpest surface plasmon peak at 520 nm

(Figure 2.1c, Figure 2.4) with a very small hydrodynamic size ca 7.7 nm and +37 mV zeta potential (Table 2.1), indicating an excellent balance achieved under these conditions. This is remarkable since bPEI 1.8kDa is a relatively short chain to provide stabilization^{17, 21} and advantages since short chain PEIs are known as less toxic than high molecular weight PEIs.

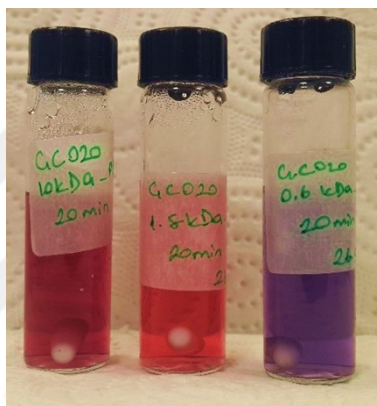


Figure 2.4: Photos of PEI10-Au, PEI1.8-Au and PEI0.6-Au after synthesis (no pH adjustment).

Lastly, we have studied the influence of the bPEI/Au mass ratio. Here, all GNPs discussed were produced at bPEI25/Au ratio of 1:7. Aggregation of nanoparticles at higher ratios (PEI/Au ratio 1:4) are reported.²¹ Using a bPEI25/Au ratio of 1:3.5 at constant bPEI25 amount caused a significant drop in the absorbance (Figure 2.1d) but a much sharper peak. The former is possibly due to lower concentration of GNP produced and the latter is due to narrower size distribution, which correlates well with the significantly lower PDI in hydrodynamic size (Table2.1).

The X-ray diffraction pattern of GNPs (Figure 2.5a) have peaks at 38.3, 44.4, 64.7, 77.6, and 81.8 degrees corresponding to (111), (200), (220), (311) and (222) planes of the face-centred-cubic (FCC) crystalline gold structure.^[132] The crystal size of PEI1.8-Au was calculated as 6.2 nm from the XRD data using the Scherrer equation. TEM images of PEI1.8-Au (Figure 2.5b) and PEI25-Au (Figure 2.5 c) indicate that GNPs are spherical with an average crystal size of 6 nm $\pm$ 3 nm. The average crystal

size measured from the TEM images support the size calculated by XRD and the hydrodynamic size measured by DLS. However, a large size distribution is observed in TEM images, which supports the relatively large PDI measured by DLS.

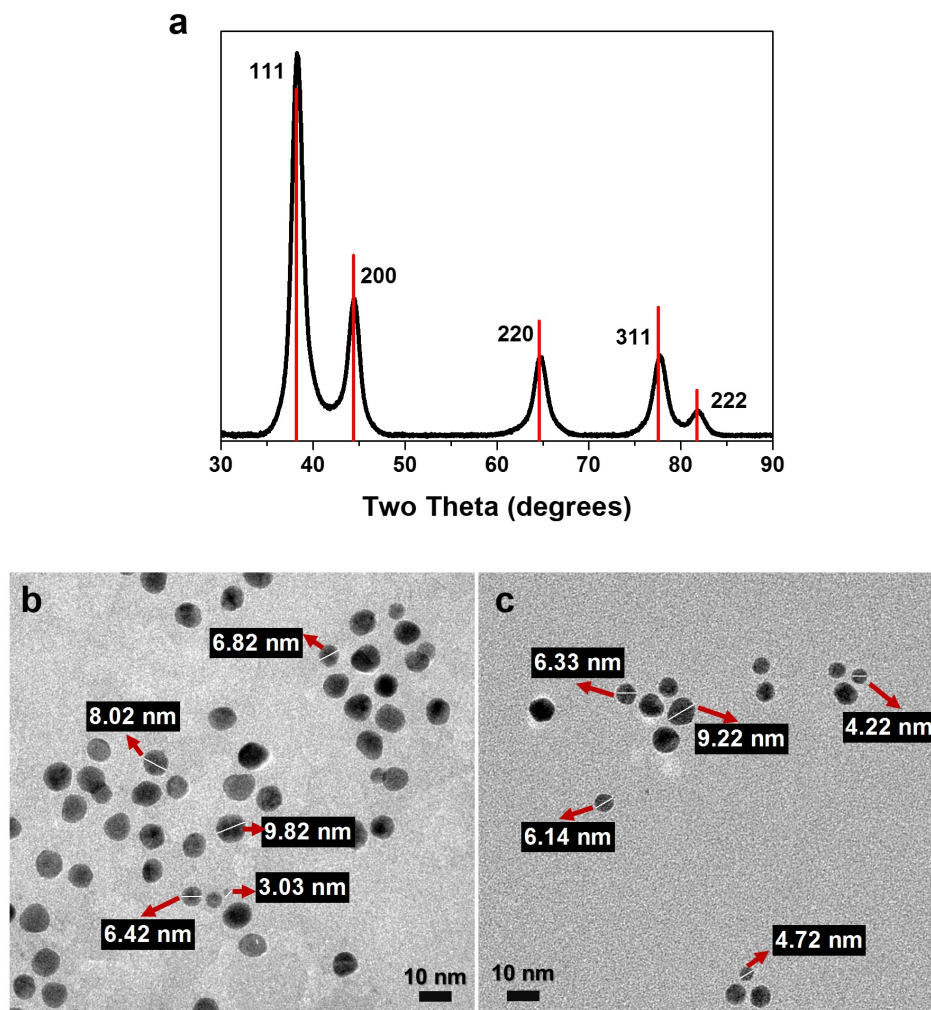


Figure 2.5: a) X-ray diffraction pattern of GNPs (PEI1.8-Au) prepared at 7:1 (Au:PEI) mass ratio synthesized at pH 9.0. TEM images of b) PEI1.8-Au synthesized at pH 9.0 and c) PEI25-Au synthesized at pH 9.0.

Cationic GNPs in such small hydrodynamic sizes would be valuable in gene transfection applications as discussed before. In order to evaluate such potential, basic toxicity and gene transfection experiments were performed. A routine MTT assay, which is based on the mitochondrial activity of viable cells, were used to determine

the toxicity of PEI1.8-Au and PEI25-Au in a dose range of 1-32 $\mu\text{g}/\text{ml}$ after 24h incubation of HEK 293T cells with GNPs. PEI1.8-Au started to show some toxicity at 4 $\mu\text{g}/\text{ml}$ and then a dose dependent toxicity between 8-32 $\mu\text{g}/\text{ml}$. Yet, the viability of cells remained above 80% within the whole dose range (Figure 2.7a). PEI25-Au presented a similar behaviour except at 32 $\mu\text{g}/\text{ml}$ dose where the viability dropped below 70%. There is no statistical difference between the toxicity of PEI25-Au and PEI1.8-Au on HEK 293T cells. One should also consider that the PEI25-Au has slightly higher organic content than the PEI1.8-Au (63% and 57%, respectively) (Figure 2.6). Overall, these GNPs are quite cytocompatible under the studied conditions, except the highest dose. The viability of bPEI treated cells in comparable doses to the organic content of the GNPs are all above 80% (Figure 2.8). Hence, since tested doses are not in highly toxic range, observation of no significant effect of bPEI molecular weight on the toxicity is reasonable.[133, 134, 135]

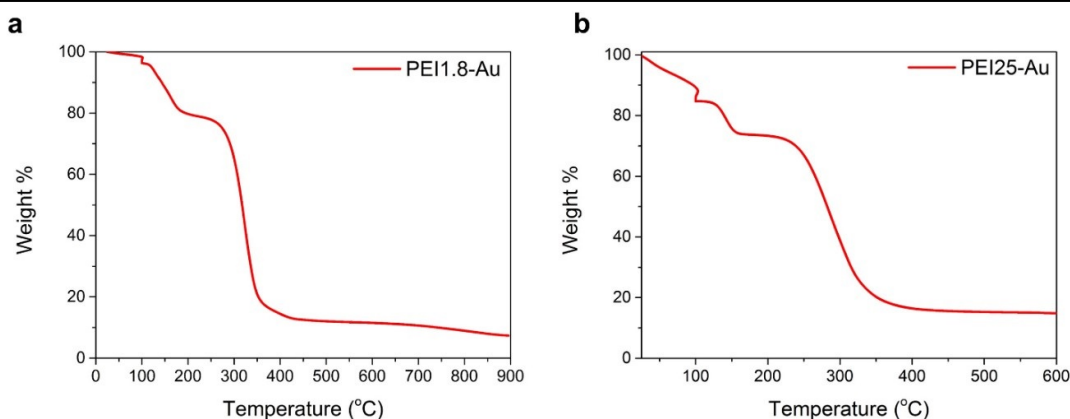


Figure 2.6: TGA graphs of a) PEI1.8Au synthesized at pH 9, b) PEI25-Au synthesized at pH 9. Weight loss at 100 °C is due to the water loss during the isothermal step.

Next, PEI25-Au and PEI1.8-Au were used to transfect HEK 293T cells with pMax-GFP vector (Lonza) DNA. FuGeNe HD transfection reagent was used as the positive control at 3/1 ratio (transfection reagent/ pDNA), as recommended by the producer. Untreated cells were considered as negative control. GNPs were loaded with the pDNA at variable N/P ratios (1/1 to 32/1) at constant pDNA amount (2.33 mg/mL)

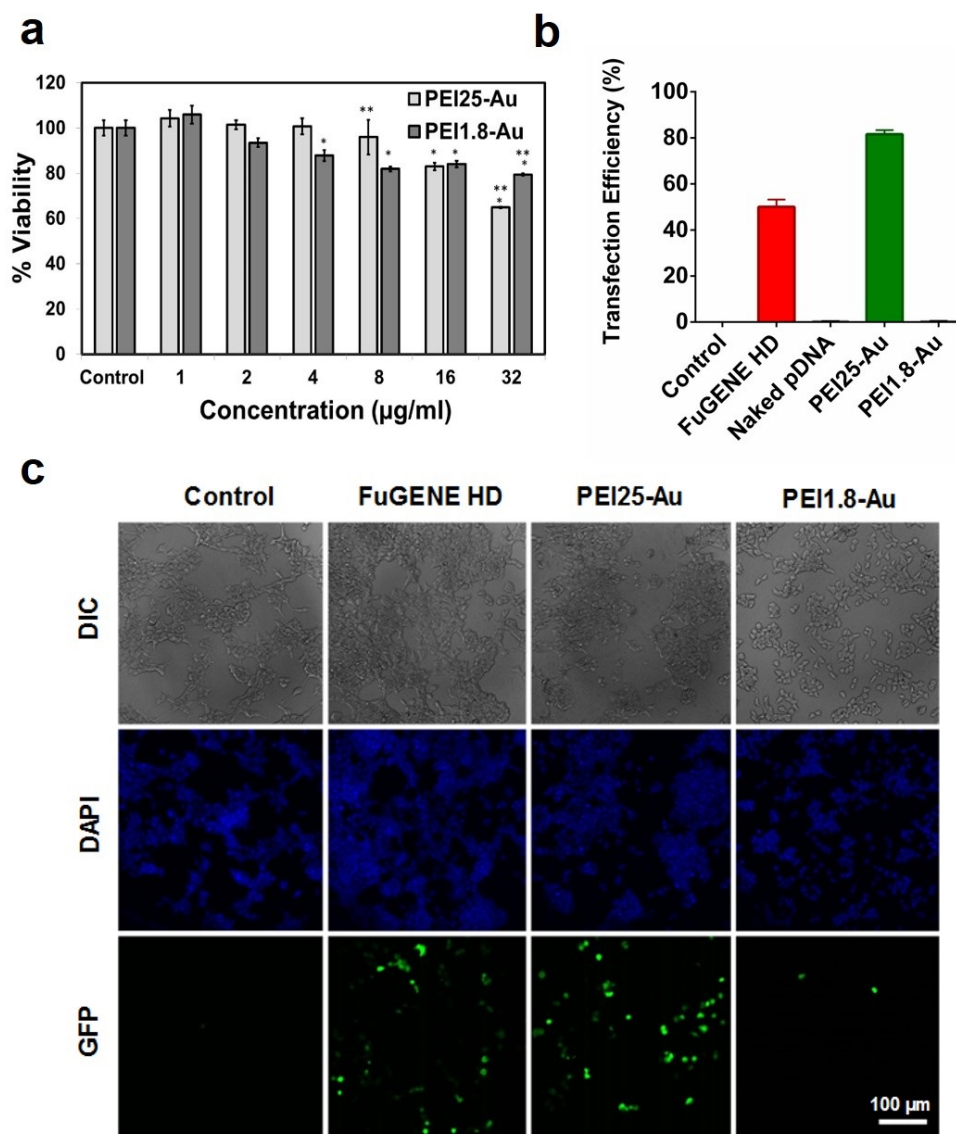


Figure 2.7: Viability of HEK 293T cells treated with GNPs. Error bars: $\pm$ SD (n=4), $p < 0.05$. (*) and (**) indicate statistically significant difference from the control and from the 16 $\mu\text{g/mL}$ dose, respectively. b) GFP transfection efficiency in HEK 293T cells, 48 h post-transfection with pMax-GFP/GNPs at 8/1 N/P ratio (8 $\mu\text{g/mL}$ GNPs/2.33 $\mu\text{g/mL}$ pDNA) as measured by flow cytometry. Untreated cells and FuGENE HD were used as a negative and positive control, respectively (mean $\pm$ SD of independent experiments, n= 3). c) Corresponding fluorescence microscopy images of (b). DIC is differential interference contrast images of the cells. Blue colour shows DAPI stained nucleus.

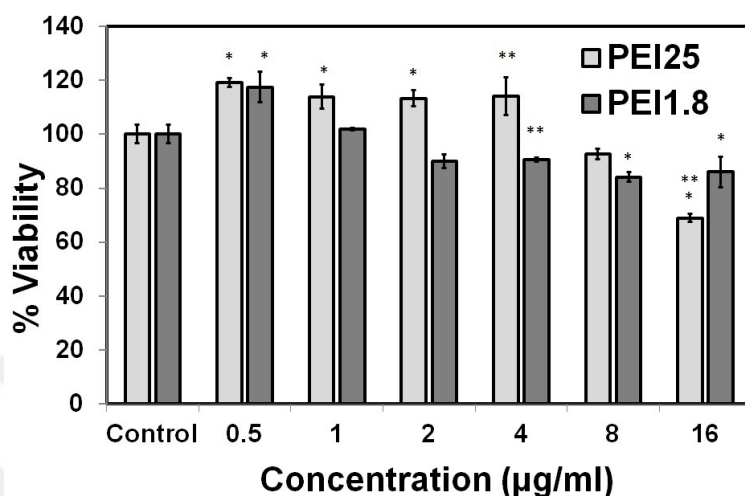


Figure 2.8: Dose dependent viability of HEK293T cells treated with bPEI 25kDa and bPEI 1.8kDa as measured by MTT assay after 24 h incubation with the polymer. Each column represents the mean with error bars representing $\pm$ SD ($n=3$), $p<0.05$. * shows statistically significant difference from the control, and ** shows statistically significant difference from the 8 $\mu\text{g/ml}$ dose.

and introduced to cells between 1- 32 $\mu\text{g/ml}$ GNP doses. DAPI images taken 48 h after transfection indicate that there is a higher density of viable cells when treated with pDNA/PEI1.8-Au with respect to pDNA/ PEI25-Au at 32 $\mu\text{g/ml}$ dose (Figure 2.9(a-b)). This is in agreement with the cytotoxicity of GNPs reported in Figure 2.7a and the literature.[131, 136] Based on these data, 8 $\mu\text{g/ml}$ GNP was determined as the highest safe dose for the transfection studies. Transfection efficiency was assessed qualitatively based on the GFP signal by the fluorescence microscopy and N/P ratio of 8/1 found as the optimal ratio (Figure 2.9(a-b)). A quantitative analysis of the transfection efficiency was done with flow cytometry at this optimal dose and N/P ratio. We have achieved 81.6 and 0.15% transfection efficiencies with PEI25- Au and PEI1.8-Au, respectively (Figure 2.7b-c). PEI25-Au performed even better than the FuGENE HD which provided 50% transfection efficiency (Figure 2.7(b-c)). Only 0.23% transfection was achieved if naked DNA was used, confirming one more time the significance of using a delivery vehicle. Inefficiency of PEI1.8-Au in transfection is in agreement with the general behaviour of low MW PEI.[133, 137]

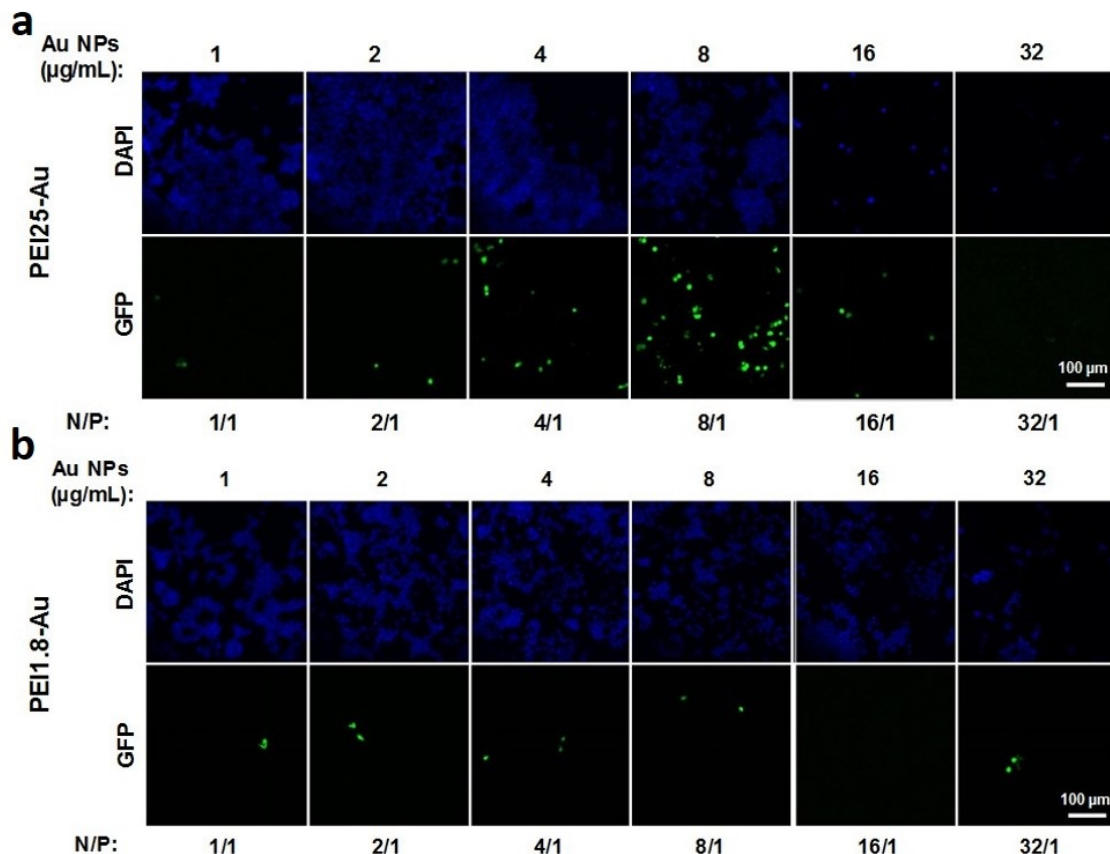


Figure 2.9: Inverted fluorescence microscopy images of HEK 293T cells after 48 h post-transfection with pMax-GFP vector by (a) PEI25-Au (b) nanoparticles at different N/P ratios. pDNA was used at fixed concentration ($2.33 \mu\text{g/mL}$). Cell nuclei were stained with DAPI (blue color).

2.4 Conclusions

In conclusion, direct reduction of auric acid with bPEI (0.6-25kDa) in water at a mild temperature does successfully produce small, cationic, colloidal GNPs. pH study performed in a controlled manner for the first time indicates that basic pH provides better reduction and surface binding and hence should be the preferred reaction pH. It is also shown that low MW bPEI is also successful in Au reduction, but PEI 0.6kDa does not provide enough stabilization to the GNPs at studied stoichiometry. Yet, PEI 1.8kDa produces quite small and stable cationic GNPs. bPEI MW does

not influence the core size as much as it influences the hydrodynamic size, hence the agglomeration. Size uniformity can be improved with increasing amount of bPEI. The oxidation of bPEI during the reduction of auric acid or binding to Au surface did not hamper the transfection efficiency of PEI25-Au and indeed it showed better efficiency than FuGENE HD. Although, PEI1.8-Au did not show an appreciable transfection efficiency, such small cationic GNPs may be used in alternative applications such as drug delivery, targeted therapy and catalysis.

Chapter 3

CATIONIC GOLD NANOPARTICLES SYNTHESIZED WITH USING LINEAR PEI

3.1 One-step aqueous synthesis of colloiddally stable, cationic gold nanoparticles using linear polyethyleneimines and their catalytic activities on methyl orange and methylene blue degradation

Gold nanoparticles (GNPs) are one of the most attractive and extensively studied materials of nanotechnology due to their unique size and shape dependent optical and electronic properties coupled with inert nature, high molar absorption coefficient and very high chemical and physical stability.[16, 17, 83, 84, 85] They are investigated as catalysts, inks, conducting materials, sensors etc.[24, 58, 59] GNPs have been extensively utilized as catalysis in different applications such as cyclization, C-C coupling, oxidation and reduction including reduction of azo dyes.[86, 87, 88, 89, 138, 139] Due to inert and nontoxic nature, they are frequently the preferred nanomaterials for medical applications, as well. This may involve imaging and/or therapy such as photothermal therapy or drug/gene delivery.[17, 60, 61, 140, 115, 119, 116, 141, 118, 142] Aqueous, stable colloidal GNPs that are not heavily aggregated are needed for medical applications. Turkevich method, the most widely used procedure to synthesize colloidal GNPs, produces large hydrophilic particles (8-120 nm) in aqueous media.[27] For further applications, particles need to be capped with stabilizers for functionalization and to prevent aggregation.[122] Organic coatings that are used to stabilize GNPs plays an important role on determining the size, shape, stability and function of these nanoparticles.[143] Organic coatings with amine functionality on nanoparticle surfaces are usually very attractive for attachment or adsorption of ac-

tive molecules to nanoparticles and/or further functionalization of the particle surface for specific duties. Polyethylenimine (PEI) is a popular amine-rich, cationic polymer, soluble in water at low pH or at high temperature, and widely used for a variety of applications.[144]. Its cationic nature allows complex formation with some metal ions, anionic molecules or polyelectrolytes. In medicine, it is accepted as the gold standard for non-viral gene delivery.[121] Both linear and branched PEI may be used but its activity and toxicity depend on the molecular weight and branching. Linear and low molecular weight PEIs are usually more biocompatible.[145]

PEI coated metal nanoparticles have been also used for the degradation of anionic azo dyes. Azo dyes are one of the most favorable dye families for several industries such as food, textile, paint and pharma due to their structural and color diversity, low cost, easy product coloration and excellent non-fade properties.[146, 147, 148, 149, 150] Despite all the benefits, azo dyes are classified as genotoxic and carcinogenic agents.[151, 152] Their by-products cause serious environmental problems such as eutrophication and prevention of aquatic photosynthesis because of absorbing and reflecting the sunlight in the contaminated water.[153, 154, 155, 156] Removing azo dyes from the wastewater has been a major concern for a long time. Recently, metal nanoparticles, mostly silver, are being investigated for dye degradation.[156, 152, 153, 157, 158, 159, 160] As the size of the nanoparticle decreases, the active sites on NPs increase due to large surface-to-volume ratio, thus the reaction rate increases.[86, 90] Most studies focused on GNP-based catalytic degradation of azo dyes utilizes GNPs synthesized in plant extracts, fungi or bacteria.[86, 152, 156, 159, 161, 162] However, controlling particle size, shape, stability and size-distribution of biosynthesized GNPs is challenging. Yet, the stabilizing agent of GNPs plays an important role on those properties and activity/selectivity of catalyst on substrates due to the attractive or repulsive forces.[143] PEI coated metal nanoparticles have been used to degrade the anionic azo dyes. Signori and Santos et al used PEI coated silver nanoparticles stabilized with 2-chloroethanol or 1-bromooctane to increase the particle stability and demonstrated the catalytic reduction of p-nitrophenol which is an anionic molecule.[143, 163]

Couple of examples demonstrate removal of anionic dyes with PEI. Long et al used bPEI-catechol coated superparamagnetic iron oxide nanoparticles (SPIONs) (500 nm in size) just for the selective removal of anionic dyes from a mixture of anionic-cationic dyes.[164] Lie et al modified silica nanoparticles with quaternized PEI for the adsorption of methyl orange (MO). As seen in these examples, anionic dyes or pollutants are adsorbed on cationic nanoparticles for separation or for catalytic degradation. On the other hand, anionic nanoparticles are used for cationic dyes, such as manganese oxide pyrolusite and carbon-doped TiO₂ at pH 7 were used for MB.[165, 158] It would be quite advantages to develop a catalytic system which can reduce both anionic and cationic dyes. Therefore, functionalization of GNP surface with PEI, especially linear and low molecular weight PEI (lPEI) due its safety, is quite attractive. In a few studies, auric acid was reduced by citrate or ascorbic acid and then stabilized with lPEI.[166, 167] One very simple way to achieve PEI-coated GNPs is the direct reduction of auric acid to GNPs by PEI. This was successfully shown in DMF and water with branched polyethyleneimine.[125, 168] Direct reduction of auric acid with linear polyethylenimine (lPEI) was first reported by Sun.[122] His study showed that gold nanoparticles, quasi one-dimensional aggregates and gold nanoplates can be formed by 423 Da lPEI at 60°C with the core size of the GNPs between 25 and 100 nm. Kuo et al reported that GNPs produced with lPEI coagulates and eventually precipitates.[169] Hence, stabilization of lPEI-GNPs is one of the main challenges. They have produced more stable GNPs by alkylating PEI to create polymer micelles, which prevented PEI chain entanglement. In another study, they modified lPEI with montmorillonite which immobilized lPEI by restricting conformational changes.[170] They were able to produce stable GNPs of 5 and 30 nm diameter. Kretschmer et al also reported the reduction of auric acid by lPEI in DMF at 150°C, but GNPs were aggregated during the synthesis.[125] We are interested in production of small, colloidally stable lPEI-coated GNPs in a simple and reproducible way for both medical and catalytic applications, due to the non-toxic nature of the core and wide applicability coupled with low cytotoxicity of lPEI. However, conditions realizing such particles

via direct reduction of auric acid with lPEI has not been understood, yet. In our previous study, we showed that branched PEI successfully reduces auric acid in water and produces small, stable, cationic GNPs in one step. Here, we studied the one-step synthesis of GNPs via reduction of auric acid with lPEI in water and identified the dependence of the process and particle properties to polymer molecular weight, pH and reaction time. lPEI is soluble in water at low pH or at high temperature, unlike branched PEI. Therefore, synthesis of GNPs by dissolution of lPEI by both methods was studied here. Small, stable cationic GNPs were prepared for the first time with lPEI in water. Protonation and deprotonation of PEI is a valuable variable for both reduction, surface adsorption and stability. We primarily investigated the influence of the pH, molecular weight of lPEI at constant lPEI: Au mass ratio and reaction time on particle properties. Additionally, we studied the influence of different mass ratio of lPEI/ Au at a constant molecular weight and reaction time on size and stability of GNPs. Since PEI is a polyelectrolyte, we suggest that it may be used for the degradation of both anionic and cationic dyes at appropriate pH values, which would be quite a practical advantage. Hence, we have also studied the degradation of MB (cationic dye) and MO (anionic-zwitterionic) dye at different pH values using the lPEI-coated GNPs produced with 2.5 and 25kDa lPEI.

3.2 Experimental

3.2.1 Materials

Gold (III) chloride solution (30 weight %) in dilute HCl (HAuCl_4) was purchased from Aldrich (Germany). 2.5 and 25 kDa linear polyethyleneimine (lPEI) were purchased from Aldrich (Germany). Centrifugal filters with 30 kDa cut-off membranes were purchased from Sartorius. Sodium borohydride (NaBH_4) was purchased from Merck. Methyl orange (MO) was purchased from Aldrich (Germany). Methylene blue (MB) was purchased from Aldrich (Germany).

3.2.2 Instrumentation

UV-vis absorption spectra were recorded on a Shimadzu UV-3600 spectrophotometer. To estimate particle size, bright field transmission electron microscopy (TEM) images were taken on a Technai G2 F30 brightfield high resolution (HR) TEM (acceleration voltage = 200 kV) using samples deposited on carbon coated Cu-grid from dilute solutions at UNAM-Ankara. To evaluate faceting, high-resolution TEM (HRTEM) imaging was performed using 200 keV electrons in a transmission electron microscope with a spherical aberration-corrected probe (JEOL JEM-ARM200CF). A Malvern Zetasizer Nano-ZS was used for determining hydrodynamic size by dynamic light scattering (DLS) and for the measurement of zeta potential. For XPS and XRD analysis, lPEI-Au solutions were freeze-dried to obtain samples in the powder form. XPS analysis were performed on a Thermo Scientific K-Alpha XPS with Al K monochromatic radiation (1486.3 eV). Powdered samples were put on a carbon adhesive tape. 50.0 eV pass energy was used for the scans with a flood gun with a 400 mm spot size. A base pressure lower than 910^{-9} mbar and experimental pressure of 110^{-7} mbar was achieved. The C1s peak at 284.5 eV was used as a reference. For XRD measurements, powdered samples were placed on a silicon holder and analyzed with a D8 Advance Bruker instrument with Cu K α radiation ($= 1.5406 \text{ \AA}$) between 2θ angles of 10°C and 80°C .

3.2.3 Synthesis of lPEI coated GNPs

For the synthesis, 10 mg/ml lPEI aqueous stock solution was prepared for each molecular weight. In Method-1, 10 mg lPEI was added into 1 ml deionized water then acetic acid was added drop wise until all lPEI was dissolved. In Method-II, 10 mg lPEI was added into 1 ml deionized water, immersed in a preheated oil bath at 95°C until all lPEI was dissolved. After the preparation of lPEI stock solution, 2.04510^{-4}M HAuCl_4 solution was mixed with 200 μl aqueous lPEI (10 mg/ml) solution. Reactions were performed in 20 ml glass vial with a teflon cap. Reaction mixtures were placed in a preheated (95°C) silicon oil and stirred at constant speed (330 rpm) for about 30

min. The change in the color of reaction mixture from yellow to reddish indicates reduction of gold from Au^{+3} to Au^{+1} to Au^0 . After cooling down the solution to room temperature samples were transferred into a falcon tube and centrifuged 30 min at 2500 rpm. The procedure was repeated 3 times and after each centrifugation the precipitated GNPs were removed. In order to isolate as much GNPs as possible without aggregation, aqueous GNPs were next centrifugation at 4000 rpm twice and 6000 rpm until the color of the solution turned light yellow. Precipitated GNPs were collected in a separate vial covered with an aluminum foil and diluted to initial volume. Resulting GNP dispersions were stored at 4 °CC. In order to investigate the effect of pH on GNP synthesis, separate experiments were performed via Method-II. Before placing the reaction mixture into the preheated oil bath, its pH was adjusted with 0.5M sodium hydroxide or hydrochloric acid solution and was mixed for 2 minutes at room temperature. Impact of the pH on lPEI coated GNPs after synthesis and purification was also studied.

3.2.4 Methyl Orange and Methylene Blue Degradation Catalyzed by Gold Nanoparticles

Degradation of methyl orange (MO) and methylene blue (MB) were performed with NaBH_4 under the catalytic action of lPEI25-Au and lPEI2.5-Au synthesized with Method-I. Experiments were performed at room temperature under different pH conditions, i.e. pH 3.5 and pH 7.5. Initially, 1967 μl 8.54 μM dye at pH 3.5 or pH 7.5 was added into 3ml quartz cuvette and the absorption of dye was recorded with the UV-vis spectrophotometer between 210 nm and 750 nm range. Then, 60mM NaBH_4 and 6 μg GNP at a final volume 2160 μl were added and an absorbance spectrum were taken and labeled as t=0. The time dependent change in the absorption peaks of the dyes were determined. For MO, peak at 464 nm at pH 7.5 and the peak at 500 nm at pH 3.5 were used. In case of MB the absorbance peak at 664 nm was used. Change in the absorbance was recoded with 2 min intervals. pH of dye solutions was adjusted with 0.5M sodium hydroxide or hydrochloric acid solution. Control experiments were

performed with 7.88 μM dye and 60 mM NaBH_4 with a total volume 2160 μl in the absence of GNPs. The % dye degradation was calculated by the following equation:

$$\% \text{dye degradation} = \frac{A_o - A_t}{A_o} \times 100\%$$

Where $[A_0]$ is the initial concentration and $[A_t]$ is the concentration at t time of dye. To calculate the % degradation of MO, absorbance of GNPs were normalized according to the MO absorbance at a wavelength where GNPs did not have absorbance. The recyclability of the GNPs as a catalyst were tested by adding a fresh sample of 3mM dye into the cuvette after full reduction of the first batch. When the degradation slowed down, 60 mM freshly prepared NaBH_4 was added to the same cuvette, followed by the addition of a new batch of 3mM dye in each cycle. Isothermal Titration Calorimetry (ITC) Experiments ITC experiments were performed on a Nano ITC TA Instruments at 25°C. 7.8 μM MO and MB prepared at pH 3.5 and pH 7.5 were injected into the 1ml sample cell in four separate experiments and 300 μl of 600 $\mu\text{g/ml}$ IPEI2.5-Au were loaded into the syringe. Dyes were titrated with 15 injections of 10 μl GNP with 10 min intervals under constant stirring. ITC experiments were also performed in the reverse order: 83.3 $\mu\text{g/ml}$ IPEI2.5-Au was added into 1 ml sample cell and 300 μl of 160 $\mu\text{g/ml}$ dyes at pH 3.5 and 7.5 were loaded into the syringe in separate experiments. IPEI2.5-Au was titrated with 50 injections of 5 μl dyes with 10 min interval under constant stirring. The heat of reaction after each injection was recorded.

3.3 Results and Discussion

GNPs were synthesized in homogenous solutions of auric acid and IPEI. IPEI is soluble in water, if it is acidified or heated. Both dissolution methods were used to investigate its influence on formation of GNPs and their properties. In Method-I, wherein IPEI was dissolved in an acidic pH, an aqueous 25kDa IPEI solution and an auric acid solution were mixed at room temperature (RT) at a mass ratio of 1:7 and then placed into an oil bath at 95°C for 20 min (pH=3.2). The initial mixture had a characteristic yellow color, due to the presence of Au^{+3} , that slowly became red in the

course of reaction. Electron transfer from the amine groups of lPEI to Au^{+3} reduces Au^{+3} and produces metallic gold. This transformation was also followed with UV-vis spectroscopy. The red spectrum in Figure 3.1a was collected from GNPs that were synthesized initially by using Method-I at an lPEI:Au mole ratio of 1:7. GNPs produced in an lPEI:Au ratio of 1:3.5 showed a weaker absorbance at 520 nm. Increasing the lPEI:Au ratio to 1:21 produced more GNPs, which were more monodisperse in size, showing a strong and narrower absorbance peak at 525 nm (Figure 3.1a). Therefore, the 1:21 lPEI:Au mole ratio was used in the rest of this study. The mixture of aqueous auric acid and lPEI solution (lPEI/ Au^{+3}) had an absorbance at 380 nm in both Method-I and Method-II. As the reduction was initiated, the characteristic surface plasmon band (SPB) of GNPs was observed at around 520 nm in both synthetic methods (Figure 3.1(b-c)).

Both reactions were monitored by UV-vis spectroscopy. The development of the SPB (520 nm) of GNPs produced by dissolution of lPEI via acidification (Method I), during a 4 h reaction, is shown in Figure 3.1b. Acidification of lPEI protonates some of the secondary amine groups and causes chain extension, due to intra- and inter-chain charge repulsion.[171] Moreover, because there are fewer amine groups for donating lone pair electrons to reduce Au^{+3} , smaller particles could be synthesized. Overall, it seems like such conditions successfully produce small and stable GNPs even at longer reaction times. On the other hand, GNPs produced in Method-II, had an SPB around 530-540 nm indicating larger or more aggregated particles at shorter reaction times, but the color stayed as a typical red color of GNPs (Figure 3.1c). In Method-II, although lPEI stock solution is not acidified, the lPEI/ Au^{+3} solution had an initial reaction pH of 3.8 due to the acidic nature of auric acid solution. Hence, protonation of lPEI should take place in Method-II, as well. In order to better observe the pH influence on particle formation, a series of reactions were conducted at different pH values at 95°C using Method-II, because this method did not require pH adjustment for the dissolution of lPEI. The pH of the initial lPEI/ Au^{+3} mixture was set to 3.5, 5, 7 and 9 with hydrochloric acid and sodium hydroxide, while fixing all other parameters.

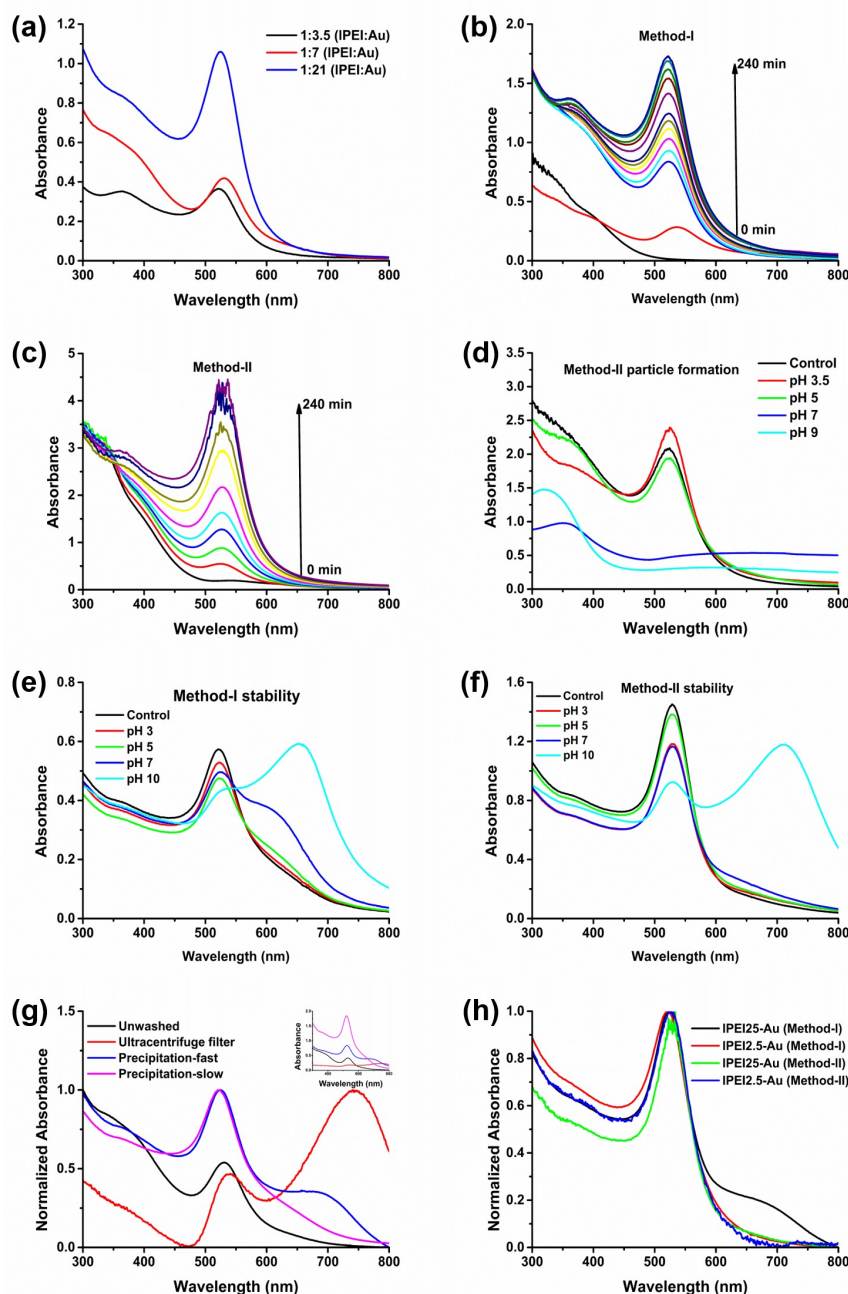


Figure 3.1: Absorbance spectrum of unwashed GNPs synthesized at different concentrations of (a) HAuCl_4 , (b) at different reaction times with Method-I and (c) Method-II, (d) at different reaction pH values with Method-II, (e-f) synthesized by different methods and after cleaning and pH adjustment, (g) GNPs before and after purification utilizing different methods, (h) GNPs synthesized with different molecular weights of PEI purified with precipitation method.

3.1 d shows the absorption spectra of these GNPs after 30 min growth in comparison with the control, which was performed without pH adjustment (original pH=3.8). GNPs were precipitated during the synthesis at pH 7 and 9. GNPs produced in controlled acidic pH (3.5 and 5) provided a slightly broader SPB, indicative of a more polydisperse size distribution, as shown in the Table 3.1. This result suggests that protonation of some amine groups is necessary for colloidal stability.

Furthermore, the influence of post-synthetic pH adjustment on GNP stability and aggregation was studied Figure (3.1(e-f)). Typical pH of the reaction mixtures was 4.2 and 5.4 for Method-I and II, respectively, at the end of the reaction, when no pH adjustment was performed in Method-II. In case of Method-I at and above pH 7 and in case of Method-II at pH 10, a second absorbance peak around 700 nm appeared due to aggregation. Deprotonation of amine groups causes interparticle H-bonding and reduction of electrostatic repulsion, therefore particles were aggregated and precipitated. Removing unbound chemicals from GNPs without causing aggregation or precipitation is always a challenge but it is rarely mentioned in the literature. Two different methods were applied for purification of the GNPs. First, GNPs were washed in a 30kDa ultracentrifuge filter with DI water at 4000 rpm for 20 min. During this process, the color of GNP changed from red to purple-blue, indicating aggregation. The absorption peak of the GNPs seen at around 750 nm after ultrafiltration supports this observation Figure (3.1g). After some elapsed time, these GNPs were precipitated. Alternatively, precipitation of GNPs via centrifugation was attempted. This approach requires a delicate control of the speed. Some aggregation was observed at 6000 rpm after 30 min. Therefore, the best way is to start the process at 2500 rpm and increase the speed slowly. The influence of the speed is clearly visible in Figure 3.1g. After optimizing the synthetic method and the washing procedure, the influence of polymer molecular weight on the particles size and stability was also studied using 2.5kDa IPEI and 25kDa IPEI. Both samples were synthesized by Method-I and cleaned with precipitation method Figure (3.1h). Even after the washing step, 2.5kDa IPEI induced the formation of more stable and smaller particles than 25kDa IPEI.

Table 3.1: Influence of pH, MW and mass ratio on the properties of GNPs

Sample name	PEI MW (kDa)	PEI:Au (weight ratio)	Size ^a (nm)	PDI ^b	Zeta Potential (mV)
Particle Formation					
Method-I					
Control-I	25	1:21	31.53	0.33	+44
lPEI2.5-Au	25	1:21	24.12	0.22	+51
Method-II					
Control-II ^c	25	1:21	25.55	0.23	+52
pH 3.5	25	1:21	52.42	0.43	+53
pH 5	25	1:21	24.32	0.45	+47
lPEI2.5-Au	25	1:21	51.42	0.25	+50
Particle Stability					
Method-I					
pH 3	25	1:21	51.55	0.44	+34
pH 5	25	1:21	46.10	0.47	+31
pH 7	25	1:21	49.27	0.47	+16
Method-II					
pH 3	25	1:21	50.50	0.46	+24
pH 5	25	1:21	41.62	0.41	+38
pH 7	25	1:21	50.97	0.52	+14

^a Number average hydrodynamic size and ^b polydispersity index (PDI) calculated from DLS measurement ^cno pH adjustment

This is quite a unique feature. Both lPEI25-Au and lPEI2.5-Au maintained their colloidal stability over 2 years after synthesis.

lPEI25-Au synthesized by Method-I was further characterized by XPS (Figure 3.2a). Two different types of N1s were observed with binding energies (BE) at ca. 399 and 400eV. The amount of bound or oxidized amines with BE at ca. 400 eV is

about 72 % of all amines. Moreover, three types of C1s with BEs at ca. 284.6, 285.9 and 287.5 eV were observed. Carbons with BE at 285.9 and 287.5 eV correspond to C-N and C=N, respectively. Formation of imide bond was also observed with bPEI in our previous study.⁵⁵ Kretschmer et al also mentioned the formation of imide during the high temperature reduction of gold with branched PEI.⁵⁶ The BE of Au 4f7 at ca. 84 eV indicates that Au is in its metallic state. The X-ray diffraction pattern of the GNPs are consistent with the face-centered-cubic (FCC) crystalline structure of gold with diffraction peaks at 38.3, 44.4, 64.7, 77.6, and 81.8 degrees corresponding to (111), (200), (220), (311) and (222) planes (Figure 3.2).[172]

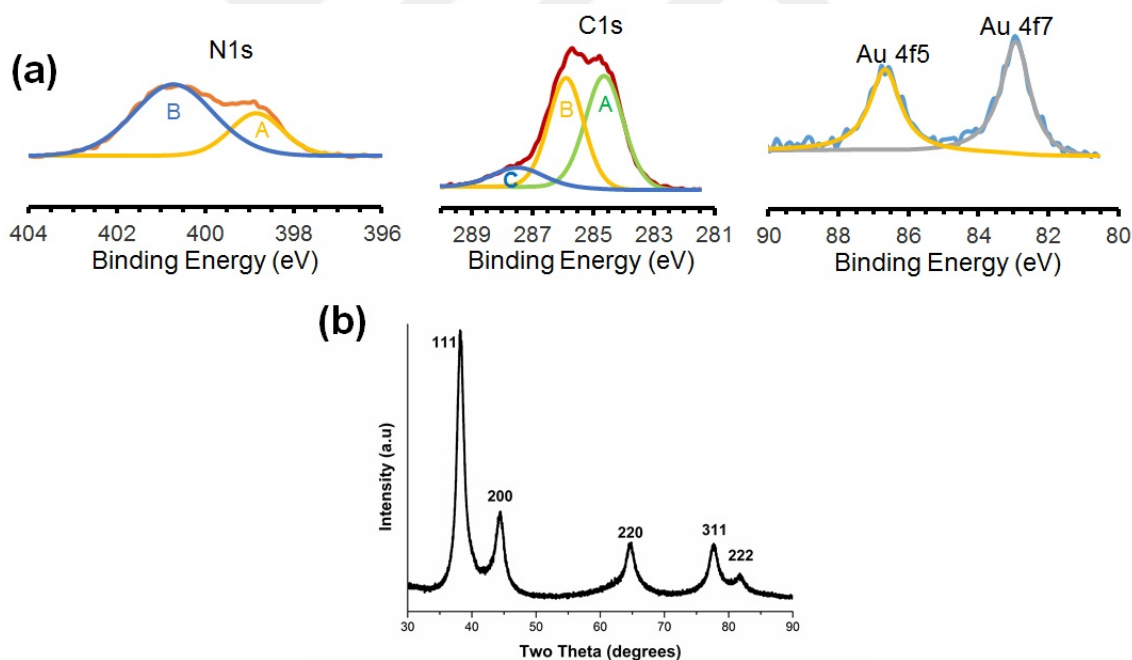


Figure 3.2: (a) XPS spectra lPEI25-Au prepared at 21:1 (Au:PEI) mass ratio synthesized with Method-I. (b) X-ray diffraction pattern of lPEI25-Au prepared at 21:1 (Au:PEI) mass ratio synthesized with Method-I.

For functionalized GNPs synthesized by Method-I (lPEI dissolved in acidic pH), HRTEM bright field imaging revealed faceted structures and suppression of faceting when the lPEI molecular weight increased from 2.5 kDa to 25 kDa (Figure 3.3(a-b)). It appears that the stretched out, shorter lPEI facilitates the ordered assembly of

gold atoms onto the evolving nanoparticle surface, allowed formation of facets with the equilibrium surface energy driven by thermodynamics. The diminished faceting tendency due to high molecular weight lPEI suggests that the kinetics of surface diffusion are more limited. Under these conditions, in which there are more amine groups available to reduce Au^3 , the higher concentration of Au^0 adsorbing on the nanoparticle surface hinders the diffusion of gold atoms to the lowest energy positions. At high resolution, facets were still observed, but to more limited extent. Delocalization effects of the coherent illumination probe can be observed in the air side of the particles. The effect of lPEI molecular weight on faceting is diminished when lPEI-functionalized GNPs were synthesized using Method-II (lPEI dissolved at 95°C). In order to estimate particle size, bright field TEM images of GNPs prepared by Method-I were taken (Figure 3.3(c-d)). lPEI25-Au nanoparticles are 14.27 ± 3.4 nm and lPEI2.5-Au nanoparticles are 15.01 ± 4 nm in diameter (Figure 3.3(e-f)).

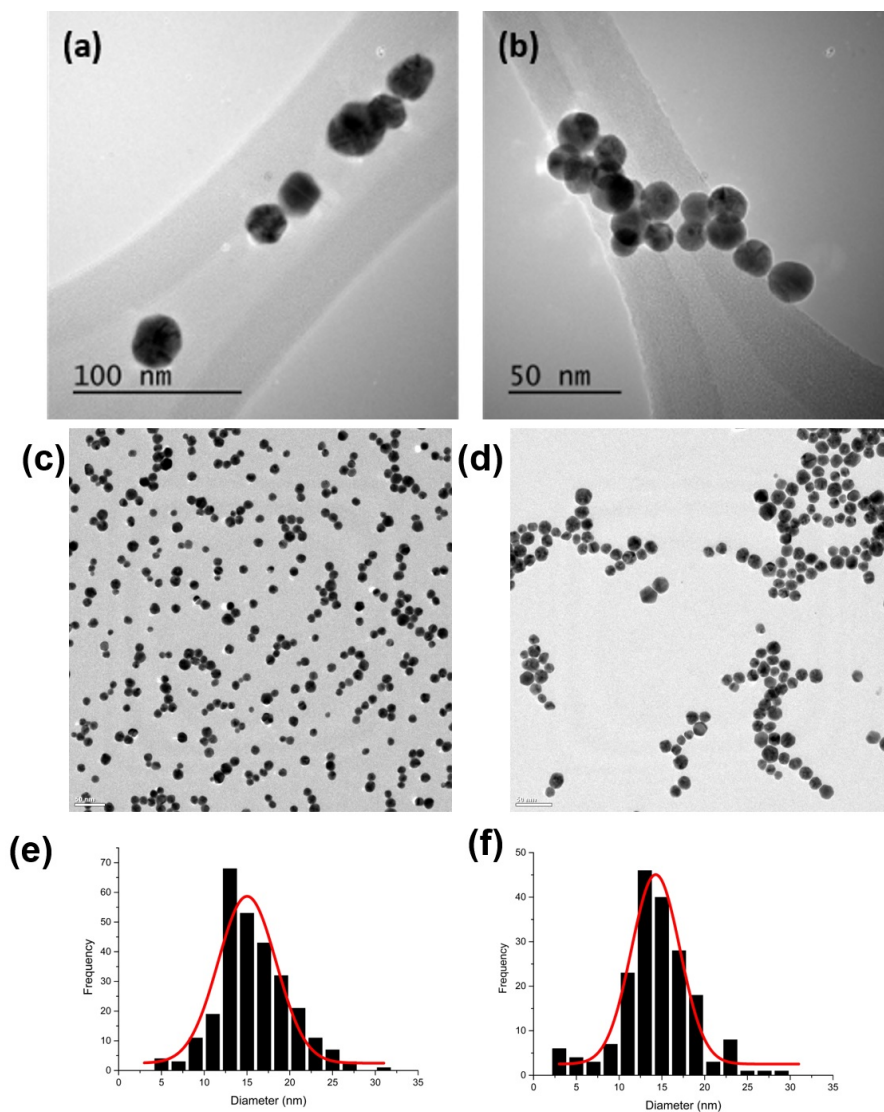


Figure 3.3: HRTEM bright field images of (a) IPEI2.5-Au and (b) IPEI25-Au nanoparticles. Bright field TEM images of (c) IPEI2.5-Au and (d) IPEI25-Au nanoparticles. Size distribution of (e) IPEI2.5-Au and (f) IPEI25-Au nanoparticles calculated from images c and d, respectively. Both samples were prepared at 21:1 (Au:PEI) mass ratio synthesized with Method-I.

3.3.1 Catalytic reduction of MO

Ability of lPEI25-Au and lPEI2.5-Au (synthesized by Method-I) nanoparticles to catalyze the degradation of MO was investigated. MO is extensively used in different areas from textile to pharmaceutical industry.[173, 174] Since it is a cancerogenic dye, its removal from the wastewater is essential. It is an anionic molecule in an aqueous media ($\text{pH} \approx 7$) but at $\text{pH} 3.5$ ($\approx \text{pK}_a$), it is zwitterionic due to the protonation of its azo functional group ($-\text{N}=\text{N}-$) as shown in Figure 3.4a. [153, 175] Therefore, we studied its degradation at $\text{pH} 3.5$ and 7.5 . In a typical experiment, $7.8 \mu\text{M}$ MO was reduced with 60 mM NaBH_4 in the presence of $6 \mu\text{g}$ GNP at room temperature. Dye degradation was monitored from the changes of the MO absorption peak as shown in Figure 3.4b for MO degradation at $\text{pH} 7.5$. The blank spectrum represents the absorbance of MO at $\text{pH} 7.5$. Absorbance recorded after addition of $33 \mu\text{l}$ 60 mM NaBH_4 was considered as $t=0 \text{ min}$ and the change in the peak intensity of the dye was recorded every 2 min for total of 30 min in the absence of GNPs. No MO degradation was detected. Although NaBH_4 is a mild reducing agent, its aqueous solution is incapable to degrade the dye molecule efficiently without a catalyst.12, 45 Addition of $6 \mu\text{g}$ GNP initiated the reduction by facilitating the electron transfer from BH_4^- to the dye molecule. The degradation lasts until BH_4^- is consumed. In Figure 3.4(c-f), degradation of MO performed at $\text{pH} 3.5$ and 7.5 with both lPEI25-Au and lPEI2.5-Au is shown with the normalized absorbance graphs and the absorbance spectrum (as the inset) between 210 to 700 nm . The absorbance peak of MO is at 465 nm at $\text{pH} 7.5$ and at 500 nm at $\text{pH} 3.5$ due to the protonation of azo group. These absorbance peaks of MO disappeared because of the cleavage of azo functional group ($-\text{N}=\text{N}-$) and a new peak at 243 nm appeared indicating formation of degradation products. In addition, a new absorbance peak around $600\text{-}700 \text{ nm}$ was observed in all four graphics which is interpreted as aggregation of GNPs. This is in the agreement with the aggregation of GNPs detected at $\text{pH} 7.5$ (Figure 3.4(c-f)). Yet, these results indicate that such aggregation does not hinder the catalytic activity of GNPs in MO degradation. Normally, we do not expect aggregation of GNPs

at pH 3.5 due to strong cationic surface charge, however, a small aggregation is suggested by the small absorbance at around 600-700 nm again. The adsorption of MO (zwitterionic at this pH) and may be degradation products on GNPs may cause secondary interactions resulting in aggregation. Overall, GNPs managed to reduce MO at both pH values, successfully but MO degradation was faster at pH 7.5 than pH 3.5 with both GNPs. Even though GNPs interact with methyl orange electrostatically, at pH 3.5 protonation of most secondary amines of IPEI may reduce the lack of electron transfer from BH_4^- to MO. MO degradation amount was calculated from the normalized absorbance data. Figure 3.4(g-h) shows that the degradation of MO at pH 7.5 was 91% with IPEI25-Au and 83% with IPEI2.5-Au right after addition reaching to total degradation of 90% in 12 min. On the other hand, the degradation of MO at pH 3.5 was 65% with IPEI25-Au and 76% with IPEI2.5-Au. This may be at least partially due to some aggregation of the former particle.

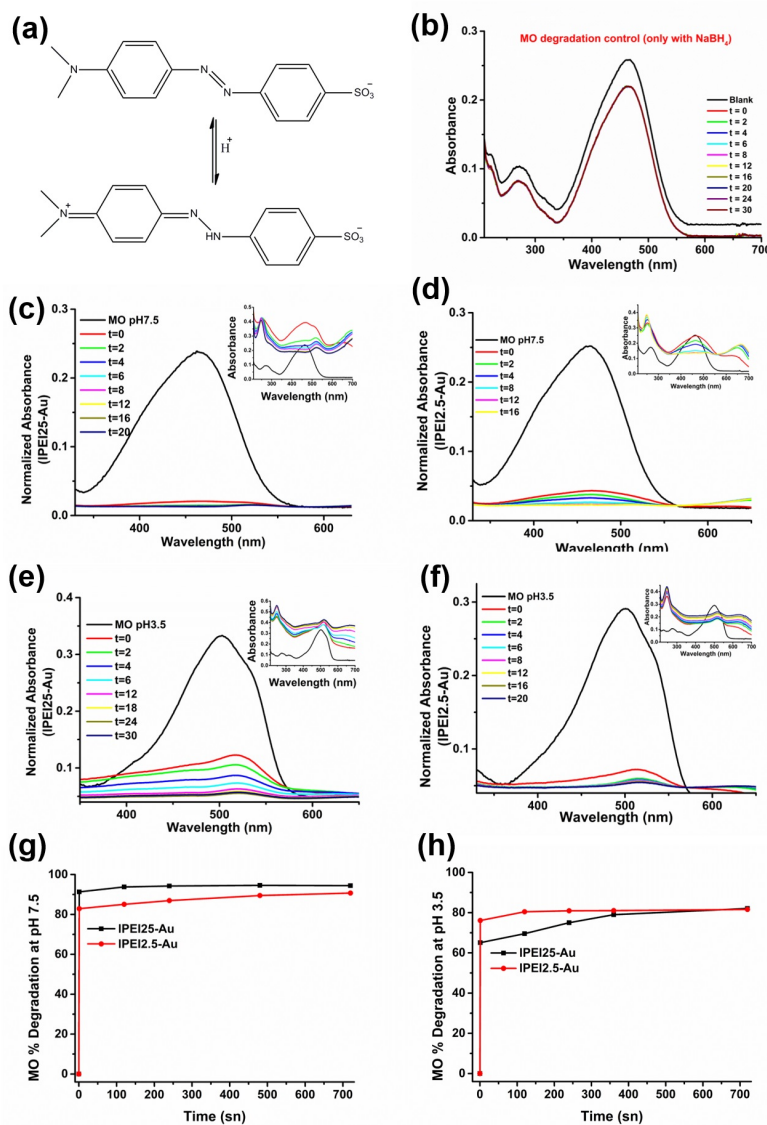


Figure 3.4: (a) Ionization of MO under acidic conditions. UV-vis absorption spectrum of time dependent degradation of MO (b) in the absence of GNP. Normalized UV-vis absorption spectra of time dependent degradation of MO between 350-650 nm (c-d) at pH 7.5 with lPEI25-Au and lPEI2.5-Au, (e-f) at pH 3.5 with lPEI25-Au and lPEI2.5-Au (Inset shows the absorbance spectra between 210 to 700 nm). (g-h) % degradation of MO at pH 7.5 and pH 3.5 with lPEI25-Au and lPEI2.5-Au.

3.3.2 Catalytic reduction of MB

Methylene blue (MB) is a cationic thiazine and is another widely utilized dye in chemical and medicinal industry. [176, 177] The resonance structures of methylene blue are presented in Figure 3.5a. Similar to MO, NaBH_4 alone was not sufficient to reduce MB (Figure 3.5 b) in appreciable amounts in the absence of GNPs. MB has absorbance peaks at 290 and 664 nm with a shoulder at 612 nm due to π to π^* and n to π^* transitions. As seen in Figure 3.5(c-f), upon addition of GNPs to MB/ NaBH_4 reduction of dye starts quickly as indicated by the reduction in the intensity of the absorbance peak 664 nm and the appearance of a new peak at 257 nm attributed to the formation of leuco methylene blue. [159] Absorbance at 600-700 nm during the reduction is again interpreted as small amount of GNP aggregation. Both MB and GNPs are cationic. MB is cationic both at pH 7.5 and 3.5. The cationic charge on GNPs are higher at pH 3.5 than 7.5 (Table 3.1). One may think that cationic GNPs would repel the cationic dye reducing the catalytic activity. However, Figure 3.5g shows that the degradation of MB at pH 7.5 was 91.5% with both lPEI25-Au and lPEI2.5-Au immediately after addition of GNPs to the dye. At pH 3.5 almost complete degradation of MB (99.4%) with lPEI25-Au and 97.5 % with lPEI2.5-Au were achieved as soon as the GNPs were added (Figure 3.5h). These results indicate no significant influence of lPEI molecular weight and better catalytic activity at pH 3.5. Here, MB is reduced with a cationic catalyst for the first time to the best of our knowledge. At pH 7.5 methylene blue is still cationic, yet the lPEI is partially deprotonated as suggested by lower zeta potential (Table 3.1). Hence, possible charge repulsion may be less, but such strong reduction may not be simply explained by this. Lone pair (lp) to π stacking interactions between aromatic rings of MB and lone pair electrons of lPEI may be considered as the attractive forced between MB and GNPs.[178, 179] At pH 3.5 GNPs would have a stronger charge, but cation to π interactions may take place as a favorable non-covalent interaction.

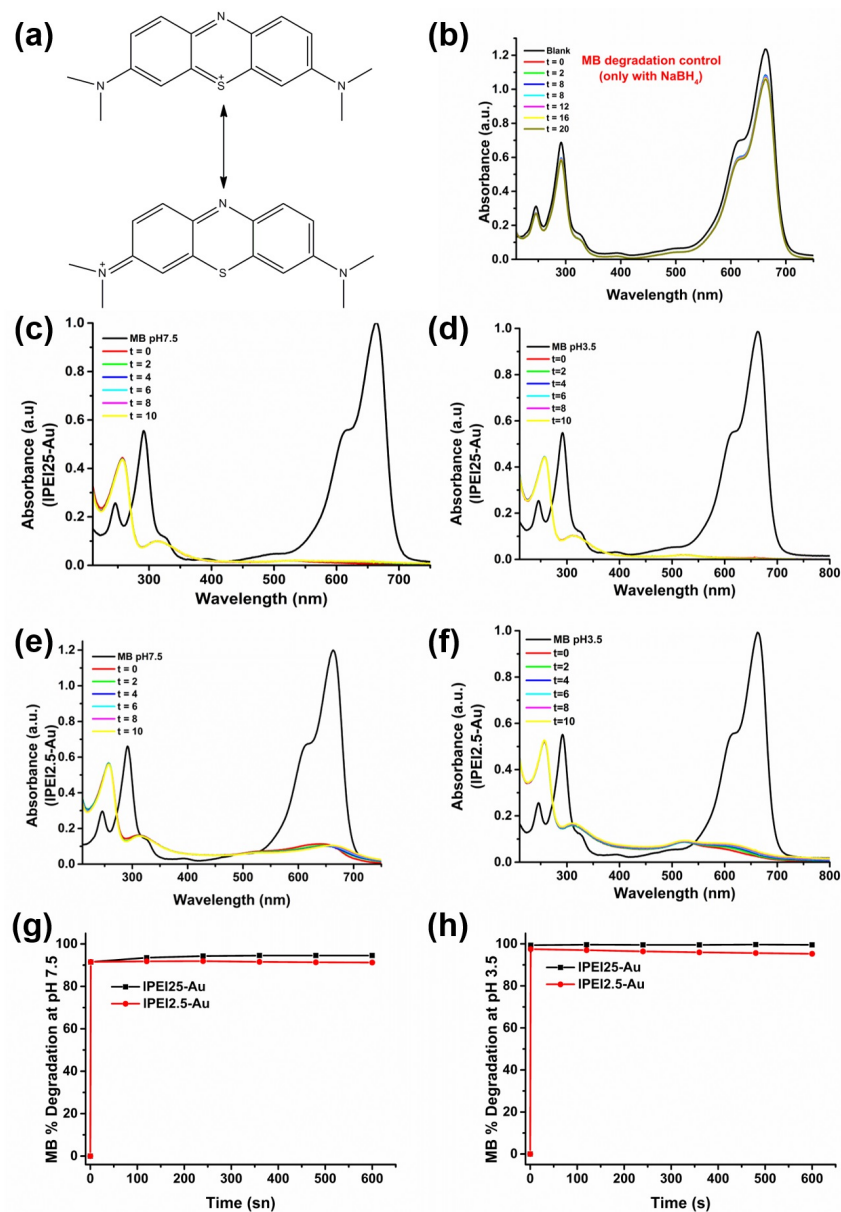


Figure 3.5: (a) Ionization of MB under acidic conditions. UV-vis absorption spectrum of time dependent degradation of MB between 210-750 nm (b) in the absence of GNP (c-d) at pH 7.5 with IPEI25-Au and IPEI2.5-Au, (e-f) at pH 3.5 with IPEI25-Au and IPEI2.5-Au. (g-h) % degradation of MB at pH 7.5 and pH 3.5 with IPEI25-Au and IPEI2.5-Au.

3.3.3 Investigation of dye-GNP interactions by ITC

The electrostatic interactions between dye molecules and GNPs were further investigated with isothermal titration calorimetry (ITC). Reaction thermodynamics and kinetics can be quantitatively determined by ITC.[180] 1 ml 7.8 μ M MO was inserted into the cell and 300 μ l 600 μ g/ml GNP were loaded into the syringe given in the Figure 3.6(a-d). Dyes (7.8 μ M) were titrated with 15 portions of 10 μ l GNPs with 10 min. After each titration an exotherm was detected indicated the binding of both dyes at both pH values (pH 3.5 and 7.5) to the GNPs.[181] Such interaction supports the degradation of both dyes at both pH values. The interaction between lPEI2.5-Au and MO at pH 7.5 was the strongest, where GNPs were cationic, and the MO is anionic. Lower binding exotherm at pH 3.5 can be explained with the charge repulsion between MO and lPEI2.5-Au. Although the interactions are weak, it seems to be sufficient to aid electron transfer from borohydride to dyes via GNPs. When the dye was titrated with gold nanoparticles, the exotherm magnitudes are not very different between MB and MO. However, if gold is titrated with the dye the difference between the interaction of MO and MB with GNPs are more visible (Figure 3.6(e-h)). Strong exotherms were detected with MO due to attractive charge interactions, as expected. On the other hand, much smaller exotherms with some endotherms were observed with the MB. This is more in line with our expectations considering the charge of the particles and the dye. Some electrostatic charge repulsion is expected which is more dramatic at pH 3.5 but also some attraction still exist between MB and GNPs, which is most probably in the form of cation- π and/or lone pair electron- π interactions, as discussed above.

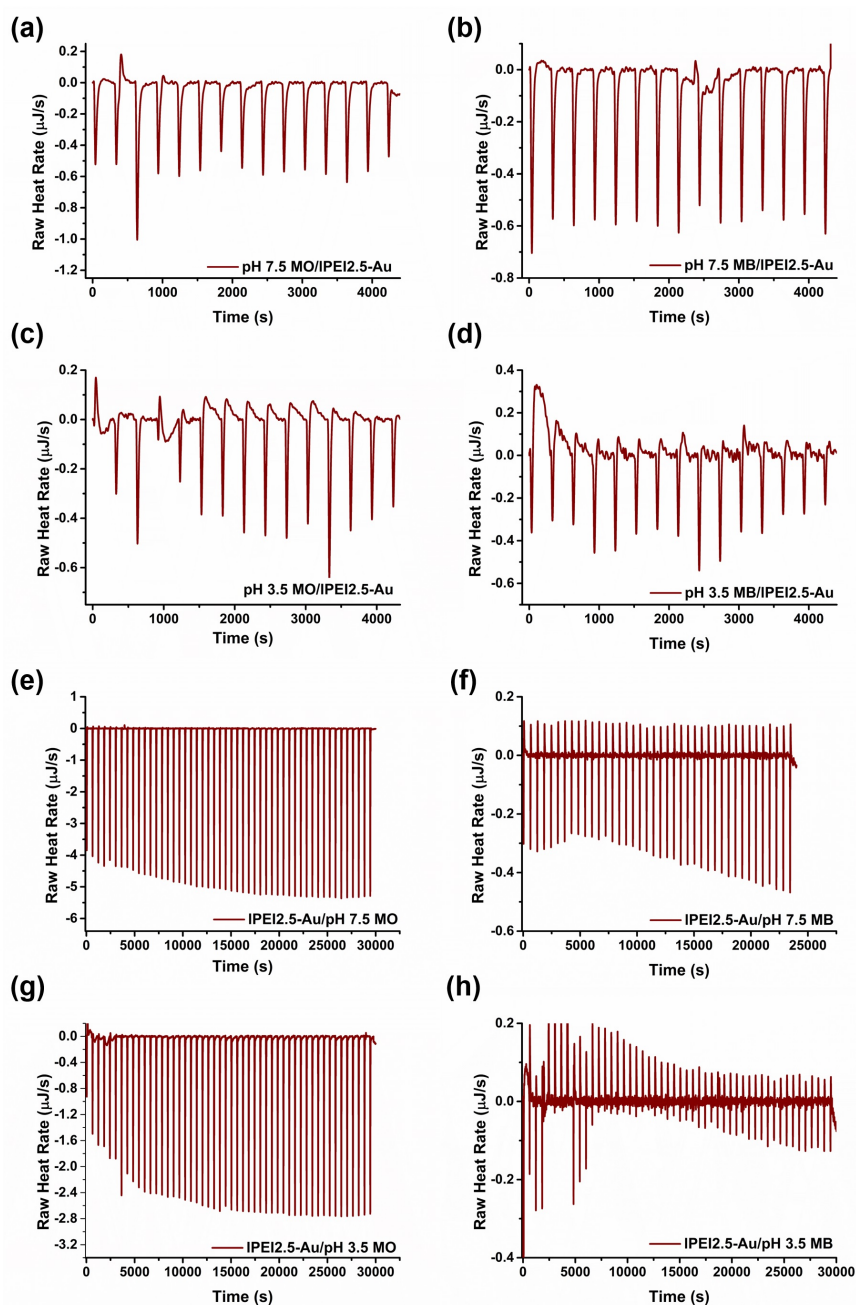


Figure 3.6: Baseline subtracted raw data for the ITC titration of (a) MO at pH 7.5, (b) MB at pH 7.5, (c) MO at pH 3.5, (d) MB at pH 3.5 with IPEI2.5-Au and titration of IPEI2.5-Au with (e) MO at pH 7.5, (f) MB at pH 7.5, (g) MO at pH 3.5, (h) MB at pH 3.5.

3.3.4 Recyclability of GNPs

The recyclability of the IPEI25-Au was tested in methyl orange degradation at pH 7.5 in the presence of 60mM NaBH₄. In each cycle, a fresh sample of the dye at the same amount was added. In the first 8 cycles degradation was around 90% (Figure 3.7a) and dropped to $\approx 60\%$ at the 9th cycle. Addition of a new batch of 60mM NaBH₄ improved the degradation to 90% again (10th cycle) indicating that the catalyst is still active. For the next 5 cycles, the activity was again around 90%. A similar experiment was performed with methylene blue at pH 3.5 and a very similar trend was observed (Figure 3.7b). These results suggest that GNPs are stable and recyclable in this process.

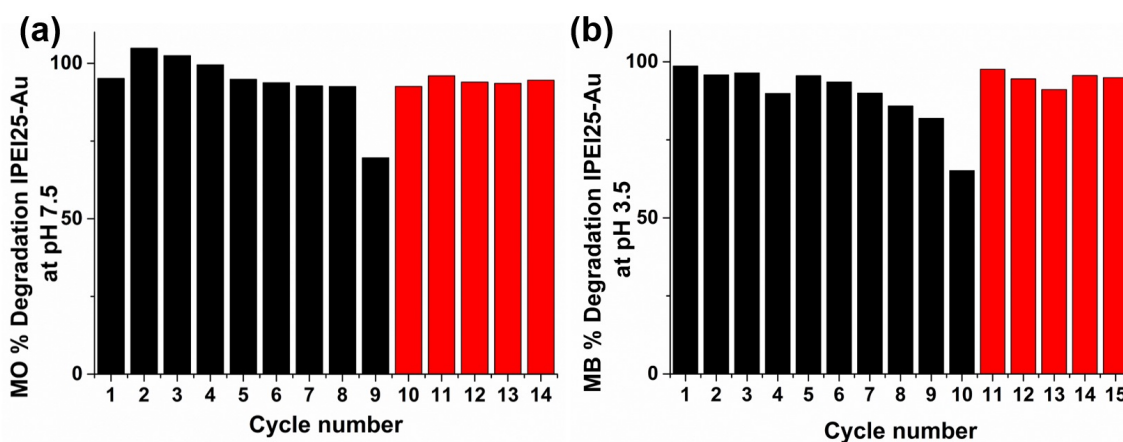


Figure 3.7: (Recyclability of IPEI25-Au on dye degradation (a) MO at pH 7.5 and (b) MB at pH 3.5.

3.4 Conclusions

Cationic gold nanoparticles (GNP) were produced by the direct reduction of auric acid in a non-reducing solvent, water, with linear polyethylenimine (IPEI), using two different synthetic protocols; one in which IPEI is dissolved by heating, and the other by acidification. The influence of pH on the particle formation and stability was studied between pH 3.5 and 10 for the first time for IPEI/GNP. This study shows

that protonation of some amine groups is necessary for colloidal stability, and it does not prevent reduction of auric acid to GNPs. This approach also made it possible to synthesize colloiddally stable gold nanoparticles in small hydrodynamic sizes with low molecular weight lPEI (2.5 kDa), as well as 25kDa lPEI. Ratio of lPEI/Au was also studied. We propose Method I, wherein lPEI was dissolved by acidification, and lPEI/Au ratio of 1/21 as the best approach and careful centrifugal precipitation procedure as a safe cleaning protocol to produce small and stable, colloidal lPEI/GNPs. For the storage purposes, we also suggest acidic pH. Although, the molecular weight of lPEI did not influence the size and the stability much, which may be surprising, HRTEM images revealed facettted crystals for 2.5 kDa lPEI coated GNPs. However, tendency for faceting decreased with increasing MW during GNP formation in acidic solutions; at elevated temperatures, the difference in GNP shape was negligible. This simple one pot synthesis of cationic GNPs in water is a valuable, simple alternative for the generation of new cationic GNPs in water with even low molecular weight PEI. These GNPs were also demonstrated as an efficient catalyst for degradation of both anionic and cationic azo dyes which is quite unique in the literature, presenting a potential as a universal catalyst. Electrostatic attraction as well as cation- π and lone pair.

Chapter 4

DEVELOPMENT OF ANIONIC GOLD NANOPARTICLES

4.1 Development of glucose decorated gold nanoparticles for transport of immunoglobulin G across the blood brain barrier

Sepsis is described as a dysregulated host response to infection which is one of the most frequent reasons of mortality in the intensive care units (ICUs). Sepsis causes multiple organ dysfunction syndrome. Sepsis induced acute brain dysfunction (septic encephalopathy) is the most common encephalopathy that has a negative influence on morbidity and mortality in the ICU. The brain dysfunction has been neglected, because there were no approved clinical or biological markers to evaluate the damage. Additionally, scientists previously believed that the brain is protected from immune activation. Recent studies have revealed that the brain is immunologically active and is one of the first organs affected, especially from the systemic inflammatory reactions in sepsis and septic shock. The brain dysfunction causes long-term cognitive impairment by leading to alterations in mental processing rate and function, memory, attention, and visual and spatial skills. The brain dysfunction destroys the integrity of the blood-brain barrier (BBB) constituted by brain capillary endothelial cells due to the cytokines and adhesion molecules that are produced extensively and released into the circulation by blood-borne immune cells and endothelial cells. Therefore, many blood-borne toxic substances easily pass into the brain. On the other hand, intact BBB prevents $\approx 100\%$ of large-molecule neurotherapeutics and more than 98% of all small-molecule drugs from entering to the brain, which makes the treatment and the development of new treatment methods very challenging. [182, 183, 184, 185, 186]. The use of intravenous immunoglobulin administration as a treatment modality for reducing sepsis-related mortality is still controversial, but experimental studies demon-

strate organ-protective effects of standard immunoglobulin G (IgG) therapy in clinical use. Kaya and his team have studied short-term and long-term protective effect of immunoglobulin (IgG and IgGAM immunoglobulins enriched with IgA and IgM) therapy in a rat model of experimental sepsis induced by cecal ligation and puncture.[187] They also reported that animals receiving IgG and IgGAM treatments under septic conditions showed less behavioral deficits, anxiety and cognitive dysfunction in the long term. The above-mentioned neuroprotective effects of immunoglobulins may be enhanced with their increasing level in the brain tissue. Yet, only 0.1% of peripherally administered immunoglobulins cross the BBB. [188, 189] Nevertheless, nanocarriers can play an important role in delivering drugs to the brain. [190] Drug loaded nanocarriers conjugated with receptor targeting molecules, can increase the transport of drugs across BBB. Targeting molecules interact with the carrier or receptor proteins on the luminal side of endothelial cells which transport nanoparticles into the endothelium and transfer to the internal areas of the brain.[191] Targeting molecules for BBB can be glucose, transferrin, low-density lipoprotein receptor-related protein-1 and l-type amino acid transporter.[192, 193, 194] Nanocarriers should be non-toxic, biocompatible with a size less than 100 nm. Additionally, they should not coagulate or get recognized by the reticuloendothelial system cells during blood circulation. [195, 196] GNPs are great candidates for delivering drugs.[197] GNPs can be coated with glucose, polyethylene glycol (PEG), transferrin, glutathione, thiamine and polysorbate-80 for protecting drugs from the defense cells in the blood, ensuring long blood circulation and drug delivery to the desired environment via molecular targeting.[198, 199, 200, 201, 202, 203, 184, 204] Polyclonal immunoglobulins have been reported to exhibit curative effects, especially in neonatal and childhood sepsis or septic shock cases.[205, 206] In the literature IgG conjugated GNPs are used for biosensors to detect certain proteins. Yet, there are no studies focused on the therapeutic effect of IgG-conjugated GNPs in vitro or in vivo, to best of our knowledge. The aim of this study was the investigation of therapeutic effect of IgG-conjugated GNPs in animal sepsis model. For this purpose, small, biocompatible nanoparticle-

based drug carrier given in the Figure 4.1 was developed. Glucose was conjugated with long PEG chain (3400 Da) for targeting the BBB. IgG was conjugated with acid-cleavable hydrazine bond using a short PEG dihydrazide (1000 Da) linker to release IgG after passing the BBB.

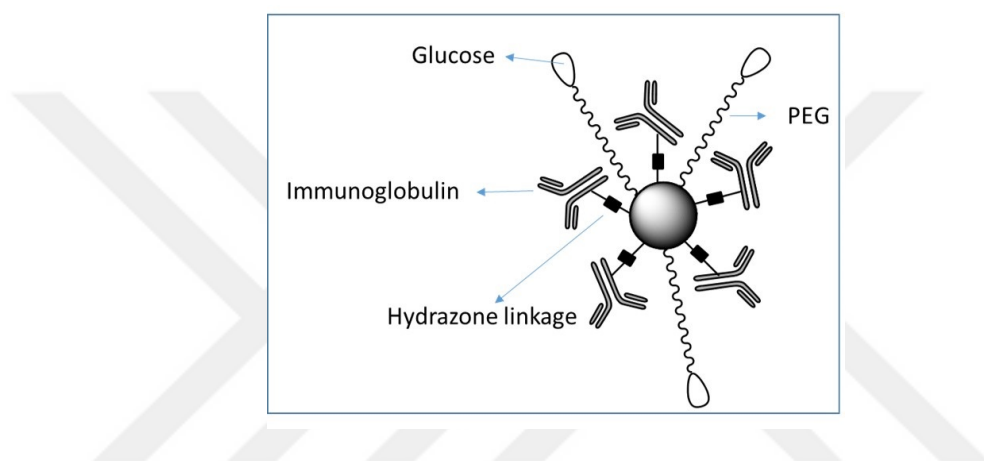


Figure 4.1: Illustration of Glucose and IgG conjugated GNP based drug carrier.

4.2 Experimental

4.2.1 Materials

Gold (III) chloride solution 30 wt % in dilute HCl (HAuCl_4), Purpald®, sodium periodate (NaIO_4) and ICP-MS (1000 ppm) Au standard solution were purchased from Aldrich (Germany). 3-Mercaptopropionic acid (3MPA) and sodium borohydride (NaBH_4) were purchased from Merck. 3400 Da maleimide-PEG- NH_2 (MI-PEG- NH_2) and 1000 Da hydrazide-peg-hydrazide (HZ-PEG-HZ) were purchased from Creative PEGworld. beta-D-Glucothiose Sodium was purchased from IRIS BIOTECH. Ethyl-3-(3-Dimethylaminopropyl)carbodiimide (EDC), N-hydroxysulfosuccinimide (sulfo-NHS) and PierceTM Coomassie Plus (Bradford) Assay Kit were purchased from Thermo Scientific (U.S.A.). Phosphate buffer saline (PBS) and 2-(N-morpholino)ethanesulfonic acid) MES monohydrate buffers were purchased from Biomatik (Canada). Centrifugal filters with 3 and 10 kDa cut-off membranes and 0.2 μm sterile cellulose acetate

(CA) filters were purchased from Sartorius.

4.2.2 Instrumentation

UV-vis absorption spectra were recorded on a Shimadzu UV-3600 spectrophotometer. To estimate the particle size, bright field transmission electron microscopy (TEM) images were taken on a Technai G2 F30 brightfield high resolution (HR) TEM (acceleration voltage = 200 kV) using samples deposited on carbon coated Cu-grid from dilute solutions at UNAM-Ankara. To evaluate faceting, high-resolution TEM (HRTEM) images taken by using 200 keV TEM with a spherical aberration-corrected probe (JEOL JEM-ARM200CF). A Malvern Zetasizer Nano-ZS was used for determining hydrodynamic size by dynamic light scattering (DLS) and for the measurement of zeta potential. Thermal gravimetric analysis (TGA) was performed by using TA Q500 to determine the organic content of nanoparticles. For TGA analysis, dried samples were heated from 25C to 600C with 10C/min rate under nitrogen atmosphere, with 30 min isothermal step at 100 C in order to vaporize the adsorbed water from the dried samples. Inductively coupled plasma mass spectroscopy (ICP-MS) was performed using Agilent 7700x ICP-MS.

4.2.3 Synthesis 3MPA coated Gold Nanoparticles (3MPA-GNPs)

GNPs were synthesized via a modified Brust-Schiffrin single-phase method. Briefly, 1.4 M, 900 μ l auric acid was added into 200ml degassed dH₂O and placed into an ice bath. Then, 1 ml 3MPA was added into the solution and mixed at 300 rpm with magnetic stirrer for 2 hours. The color of the solution was white and cloudy. Then, a freshly prepared degassed 0.625 M, 20 ml NaBH₄ solution was added into the Au-S solution fast with two 10 ml injectors at the same time. The white cloudy color was changed to brownish color. After 1 hour, the solution was poured into a 250 ml glass bottle and pH was adjusted to 7.2 and mixed 2 hours in order to increase the stability of the particles. Then, samples were washed from 3000 Da ultra-centrifugal filter at 4500 rpm. Final product was filtered with 0.2 μ m sterile CA filter. Then, 3 ml of

3MPA-GNPs was lyophilized, and 3mg/ml sample was obtained.

4.2.4 Preparation of glucose and IgG conjugated GNP (Glu-GNP-IgG)

For 5% glucose conjugation, glucose-thiol (1.35×10^{-6}) was reacted with 9×10^{-7} mole 3400 Da MI-PEG-NH₂ linker in a PBS buffer at pH 6.5 for two hours. In a separate flask, 5.46 mg (35% coating) GNPs was dissolved in 0.1 M MES buffer at pH 6. 1.35×10^{-6} mole freshly prepared EDC solution (1mg/ml) in MES buffer was added in GNPs solution and mixed for 2 min. Then, 1.35×10^{-6} mole freshly prepared Sulfo-NHS solution (1mg/ml) in MES buffer was added in the solution and mixed for 20 min. After 20 min, GNP solution was washed with 1 X PBS buffer with 3000 Da ultra-centrifugal filter at 4500 rpm three times. pH was set to 7.3. Glu-S-MI-PEG-NH₂ solution was added directly into the GNP solution and mixed 4 hours. If the product would be utilized for IgG conjugation step, the solution was washed with 0.1 M MES buffer at pH 6, if not, the solution was washed with 1 X PBS at pH 7.3.

In the previous step the final product was washed with 0.1M MES buffer. For 0.15% IgG conjugation, the following procedure was followed: 2.43×10^{-7} mole, freshly prepared EDC solution (1mg/ml) in MES buffer was added in GNPs solution and mixed for 2 min. Then, 2.43×10^{-7} mole freshly prepared Sulfo-NHS solution (1mg/ml) in MES buffer was added in the solution and mixed for 20 min. Activated nanoparticles were washed with 1 X PBS buffer using ultra-centrifugal filter (MWCO 3000 Da) at 4500 rpm three times. 2.7×10^{-8} mole freshly prepared 1000 Da Hz-PEG-Hz solution (1mg/ml) in 1X PBS was added to this solution and mixed overnight. Next day, the solution was washed with 1 X PBS buffer with 3000Da ultra-centrifugal filter at 3000 rpm three times. pH of the solution was adjusted to 9.

Parallel to these, IgG oxidation was performed before its conjugation to hydrazide functional GNPs: 4.05 mg, 81 μ l IgG from Ig Vena was mixed with 2025 μ l 0.1 M Na₂HPO₄ pH 8 buffer to obtain 2mg/ml IgG solution. In a separate vial which was covered with an aluminum foil, 100mM 1 ml NaIO₄ solution was prepared in dH₂O. 405 μ l 100mM NaIO₄ solution was added into IgG solution and incubated in the

dark for 30 min. Then the solution was washed with 1X PBS pH 9 with 10kDa ultracentrifugal filter three times. Purpald test was used as colorimetric test for IgG oxidation. 10 mg Purpald was dissolved in 1ml 1 M NaOH. 20 μ l 1mg/ml IgG was mixed with 60 μ l Purpald. The solution color was changed from clear to purple as an indication of oxidized IgG. Finally, oxidized IgG was mixed with previously prepared GNPs solutions at 4°C for two days. pH was adjusted to 9. The final product was filtered with 0.2 μ m CA sterile filter in the laminar flow cabinet. Additionally, glucose conjugated GNP (Glu-GNP) and IgG conjugated GNP (GNP-IgG) were also synthesized using exactly the same procedures.

4.2.5 Bradford Assay

The calibration curve was prepared by using known amount of IgG from Ig Vena. Briefly, 1ml 12, 10, 8, 6, 4, 2 and 0 μ g/ml IgG in pH 9, 1X PBS and 4 μ l of the final product were mixed with 1 ml Bradford assay and incubated for 5 min. Later, the absorbance of IgG conjugated GNPs at 595 nm was measured and IgG amount on GNPs were calculated according to the calibration curve.

4.2.6 Au content determination by ICP-MS

All volumetric flasks were cleaned with aqua regia before the analysis. 100 μ l of each sample were digested with 500 μ l aqua regia and incubated overnight in 10 ml volumetric flasks. Later, samples were completed to 10 ml with dH₂O and transferred into ICP-MS flasks for the measurement. The calibration curve was prepared between 0 to 7.5 ppm by using 1000 ppm Au standard solution. Au content of GNP, Glu-GNP, Glu-GNP-IgG and GNP-IgG was calculated from the calibration curve.

4.2.7 Animal studies

Animal studies were performed by Prof. Mehmet Kaya's laboratory. Animal studies were performed after obtaining the permission from Koç University Animal Ethics Committee. The procedures of local ethics committee were followed (Ethics Com-

mittee Permission No: 2018.Hadyek.028). Adult Wistar / Albino rats are used in the experiments. The animals were kept for 12 hours at dark and 12 hours at day light under normal feeding conditions. All experiments were conducted in accordance with the ethical rules. The cecal ligation and puncture (CLP) model was performed according to the literature procedure.[207]

4.2.8 Tissue biodistribution of GNPs

Tissues were collected immediately after the guillotine. All tissues were weighted and homogenized in 1 ml 1X PBS. All volumetric flasks were cleaned with aqua regia before the analysis. Homogenous tissues were vortexed before putting in 10 ml volumetric flasks. 200 μ l of tissue samples were put separately to the volumetric flasks as three replicas and heated at 120°C for 30 min. Then 1 ml of aqua regia and 375 μ l of hydrogen peroxide (H₂O₂; %30) was added. Samples were incubated for one week at room temperature (RT). At the end of the incubation time, all flasks were completed to 10 ml with dH₂O. To remove the salt produced during the tissue digestion, all samples were centrifuged at 6000 rpm for 15 min and transferred into ICP-MS flasks. The calibration curve was prepared between 0-50 ppb by using 1000 ppm Au standard solution. The Au concentration in tissues were measured according to the calibration curve.

4.3 Results and Discussions

4.3.1 Synthesis and Characterization of Functional GNPs

GNPs with a size between 5 to 80 nm were reported to cross the BBB.[199] To synthesize small and colloiddally stable and functional GNPs, 3MPA was used as the coating agent. Au creates stable covalent bond with thiol groups.[208] GNPs were synthesized via a modified single-phase Brust-Schiffrin method as shown in Figure 4.2. In the literature, modified single-phase Brust-Schiffrin method is mostly performed in methanol/dH₂O mixture.[199, 37, 36] Here, we achieved the synthesis of GNPs in

just dH₂O. Initially, HAuCl₄ was reacted with 3MPA for 2 hours under Ar in an ice bath to allow the formation of Au-S bond. Then, NaBH₄ was injected to the system for mild reduction. Because, NaBH₄ is a strong reducing agent, the temperature of the reaction should be around 0°C. After NaBH₄ reduction, the color of the solution was changed from cloudy-white to cloudy brownish. At the end of the reaction pH was 4.6 which is the pK_a value of 3MPA. As the pH increased solubility of 3MPA in water was increased and the color was turned to clear brown shown in Figure 4.3A.

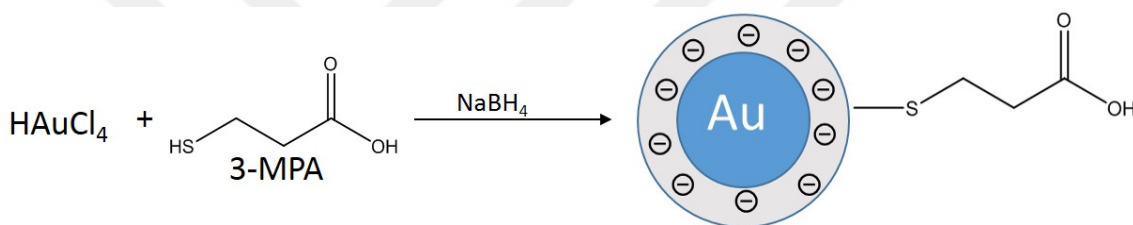


Figure 4.2: . Illustration of synthesis of 3MPA coated GNPs.

UV-vis spectrum of GNPs shows a strong absorbance at 400 nm region (Figure 4.3B). UV-vis spectrum of GNPs shows a strong absorbance at 400 nm region (Figure 4.1B). The two absorbance peaks seen at 350 nm and 385 nm in the dilute samples are characteristic Au-S absorption peaks (inset of Figure 4.3B). On the other hand, the SPR band was not observed. The reason of this is the small size of GNPs which is indeed considered as a cluster and hence there are low number of electrons to oscillate with the field and create a SPR peak.[19] TEM images of the sample given in the Figure 4.3C indicated the cluster form. GNPs have ca 2 nm core size. The organic content of GNPs was determined as 35% from the TGA thermogram given in the Figure 4.3D.

GNPs were further characterized by DLS and zeta potential. The intensity based hydrodynamic size of 3MPA coated GNPs were 21.04 nm and the number based hydrodynamic size was 11.70 nm with a PDI 0.17, which indicates relatively homogeneous size distribution. The difference between core and hydrodynamic size is probably because of the coating, some aggregation as well as bound solvent. These particles have

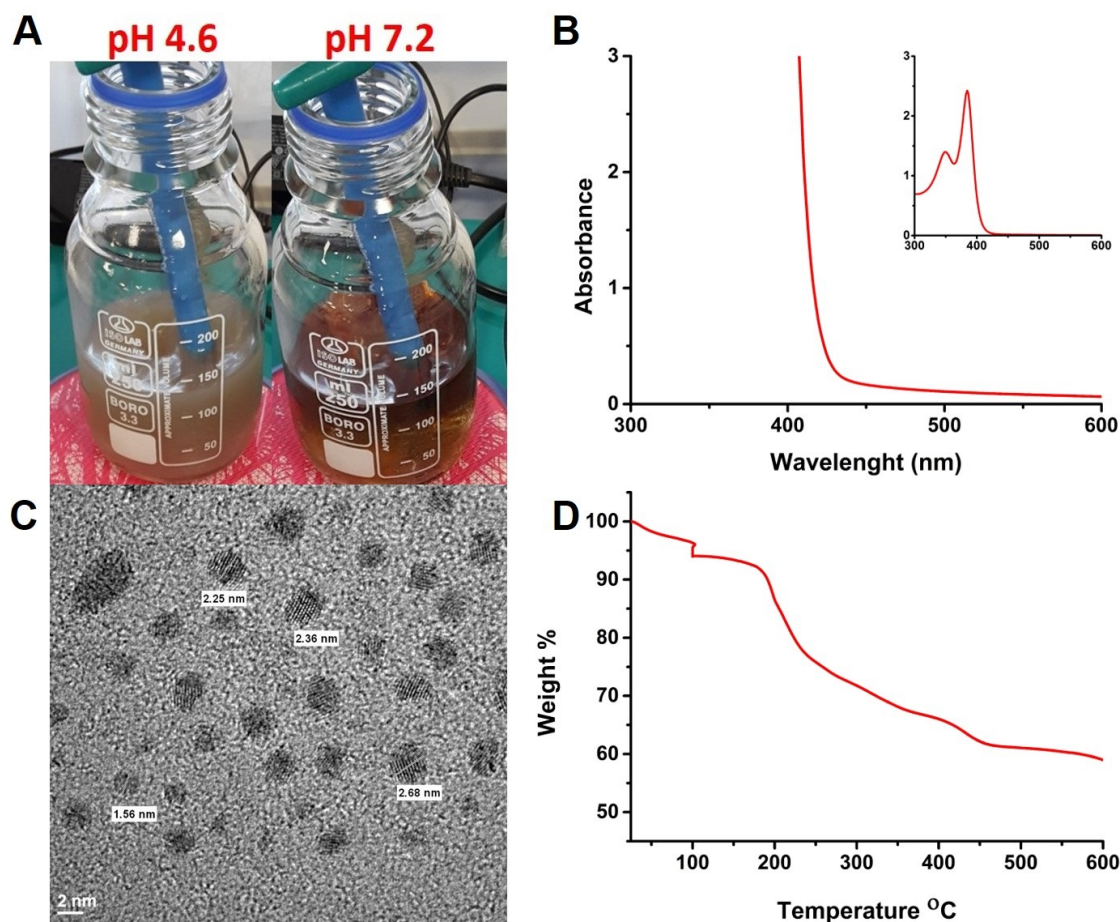


Figure 4.3: (A) Photographs of 3MPA coated GNP at pH 4.6 and 7.2. (B) UV-vis spectrum of 3MPA coated GNP taken after washing with dH₂O and completed to its initial volume after synthesis. The inset shows spectrum of diluted sample of same GNP. (C) TEM image of GNPs. (D) Thermogram of dried sample of washed GNPs.

a strong negative zeta potential (-41.2 mV) indicating anionic nature strong enough to provide electrostatic stabilization.

GNPs were carefully designed to pass BBB and to release IgG (Figure 4.1). For this, glucose and IgG conjugations to GNPs were performed carefully. Glucose conjugation was completed in two steps (Figure 4.4). The first step involved the reaction of glucose thiol with MI-PEG-NH₂ linker in a PBS buffer at pH 6.5. The pH of this step was crucial since, the reaction between maleimide and thiol is very specific between pH 6.5 and pH 7.5. Yet, it can also react with primary amine groups at higher pH

Table 4.1: Hydrodynamic size and zeta potential of 3MPA coated GNP and influence of glucose and IgG conjugation on properties.

Sample name	Size ^a (nm)	Size ^b (nm)	PDI ^c	Zeta Potential (mV)
GNP*	21.04	11.70	0.17	-41.2
Glu-GNP*	24.36	12.44	0.17	-21.6
GNP-IgG ⁺	32.67	13.54	0.32	-15.4
Glu-GNP-IgG ⁺	43.82	15.94	0.25	-13.0
IgG*	12.92	7.53	0.18	-3.32

^a Intensity based hydrodynamic size ^b Number average hydrodynamic size and ^c polydispersity index (PDI) calculated from DLS measurement.* in 1X PBS pH 7.2 and + in 1X PBS pH 9.

(pH >8.0). Since the PEG linker contains both maleimide and primary amine end groups, reaction pH was important. In the second step, GNPs were activated via typical EDC/Sulfo-NHS procedure and mixed with Glu-S-MI-PEG-NH₂ for 4 hours and washed with 1 X PBS. Unfortunately, the amount of conjugated glucose was too low to detect with commercial glucose kits.

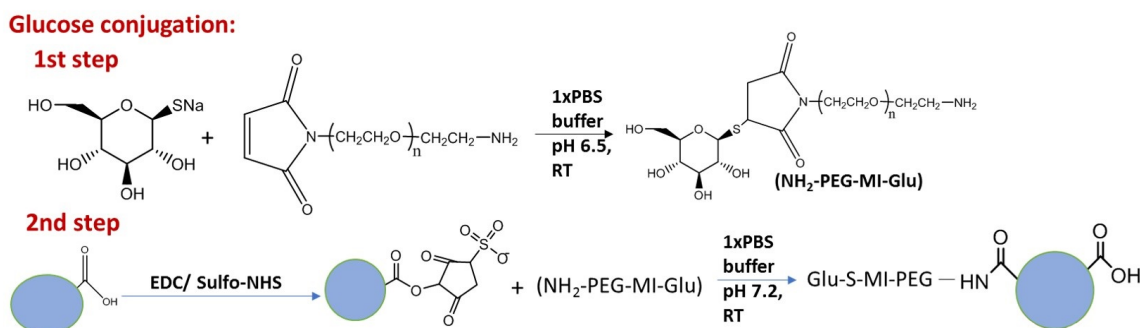


Figure 4.4: Illustration of glucose conjugation to GNP.

The challenging part of this study was the IgG conjugation to GNPs. For this study, commercial Ig Vena (5g/100ml), which is polyclonal human normal IgG pro-

duced from plasma of 1000 and 15000 donors, was used. It contains four different subclasses such as IgG1, IgG2, IgG3 and IgG4. All subclasses of IgG have different isoelectric points (pI) in the range of pH 6.3-8.9.[209, 210] Isoelectric point is curial for stability of antibodies which is directly related to pH of the environment of surrounding medium. Antibodies have positive charge below and negative charge above their pI values. At the pI value, antibody is electrically neutral which affects the solubility. Therefore, pH of buffers was one of the most important parameter for the conjugation chemistry of IgG in this study. IgG conjugation was done in three steps as summarized in Figure 4.5. The first step was the conjugation of HZ-PEG-HZ (1000Da) to glucose conjugated GNP via typical EDC/Sulfo-NHS chemistry. Acid-cleavable hydrazine functional PEG linker was used for the controlled release of IgG after passing BBB. At the end of the HZ-PEG-HZ conjugation reaction, pH was set to 9 considering the solubility of IgG.

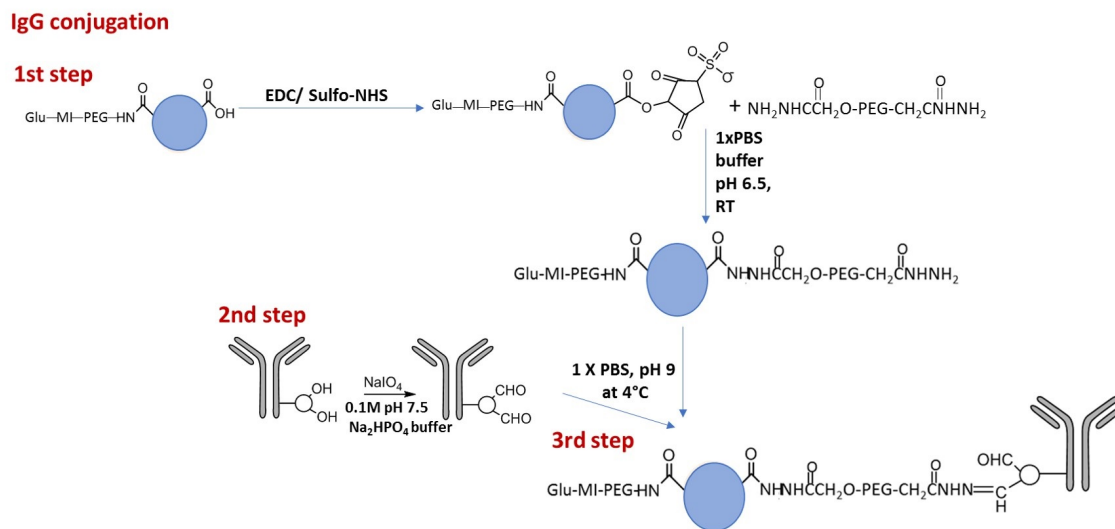


Figure 4.5: Illustration of conjugation of IgG to Glu-GNP.

The second step was the IgG oxidation. An IgG monomer is a Y shaped glycoprotein consisting of two heavy chains and two light chains linked each other by disulfide bonds (Figure 4.6). Heavy chains are found in Fab (Fragment antigen binding) fragments which is the region where antigen binds. Light chains are found in the

Fc (Fragment crystallizable) fragment which does not have antigen-binding activity. In order to preserve the antigen-binding activity of IgG, it was conjugated to GNPs from the Fc. To achieve this, carbohydrate groups in the Fc fragment were oxidized. IgG oxidation reaction was done in 0.1 M pH 8 Na_2HPO_4 buffer. Originally, the oxidation reaction takes place at pH 7.2- 7.5.[211, 212] Yet, IgG2 and IgG4 had pI value at 7.2 and 7.4, respectively. Therefore, oxidation was performed at pH 8. IgG was oxidized with NaIO_4 in dark for 30 min. Purpald test confirmed the oxidation of IgG by changing color from clear to purple as shown in Figure 4.7.

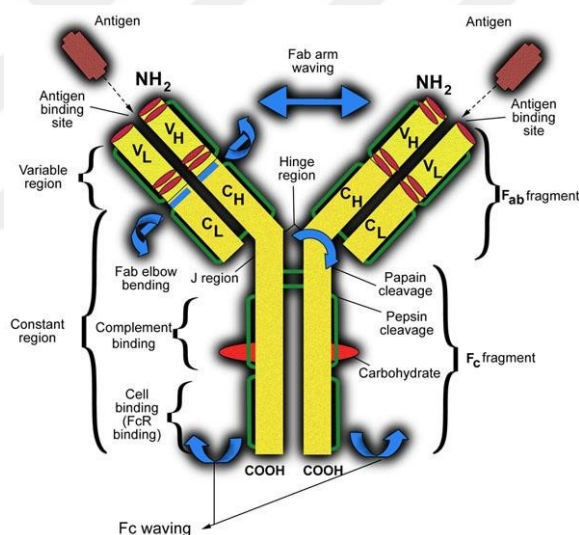


Figure 4.6: Illustration of IgG structure. [12]



Figure 4.7: Purpald test on IgG (A) before and (B) after the oxidation reaction.

Oxidized IgG (ox-IgG) was washed with 1 X pH 9 PBS buffer with 10kDa ultra-centrifuge filter for 3 times. Yet, some of IgG was bound to the filters during the washing step. The ox-IgG was mixed with GNP solution (pH 9) for 2 days at 4°C. Because IgG is the most common antibody in the immune system, it can bind to many kinds of pathogens such as bacteria, fungi and virus easily. Therefore, sterilization of the final product is another crucial parameter for the study. The final product (Glu-GNP-IgG) was sterilized by using 0.2 μm sterile CA filter under laminar hood. Only glucose conjugated GNP (Glu-GNP) and only IgG conjugated GNPs (GNP-IgG) were also synthesized for the animal studies along with Glu-GNP-IgG with using exactly the same procedures and stoichiometry.

After conjugating glucose and IgG to GNPs, size and zeta potential of all GNP samples were determined (Table 4.1). Particle size of GNPs were increased, and the zeta potential of GNPs were decreased as expected after each conjugation. Overall, the size was always less than 50 nm which is critical to pass the BBB. Additionally, PDI values indicated the monodispersity of the particles.

To calculate the IgG content of GNPs, the Bradford assay was used. Bradford assay is a simple and fast calorimetric assay for measuring the concentration of protein in a solution. First, a calibration curve was prepared by using known amount of IgG (0-12 $\mu\text{g}/\text{ml}$) from Ig Vena (Figure 4.8A). Then, diluted IgG bound GNPs were mixed with Bradford assay and their absorbance at 595 nm were measured. GNP-IgG sample contains 807.4 $\mu\text{g}/\text{ml}$ IgG and Glu-GNP-IgG sample contains 916 $\mu\text{g}/\text{ml}$ IgG.

Au concentration in the final product was determined with ICP-MS. A calibration curve was prepared from 1000 ppm Au stock solution as shown in the Figure 4.8B. IgG and Au concentrations of each GNPs were summarized in the Table 4.2. The main difference in the [Au] concentration originates from the dilution of samples.

4.3.2 *In vivo studies*

The cecal ligation and puncture (CLP) model is one of the widely used experimental sepsis model in rodents. The CLP model experiments were performed by Prof. Kaya's

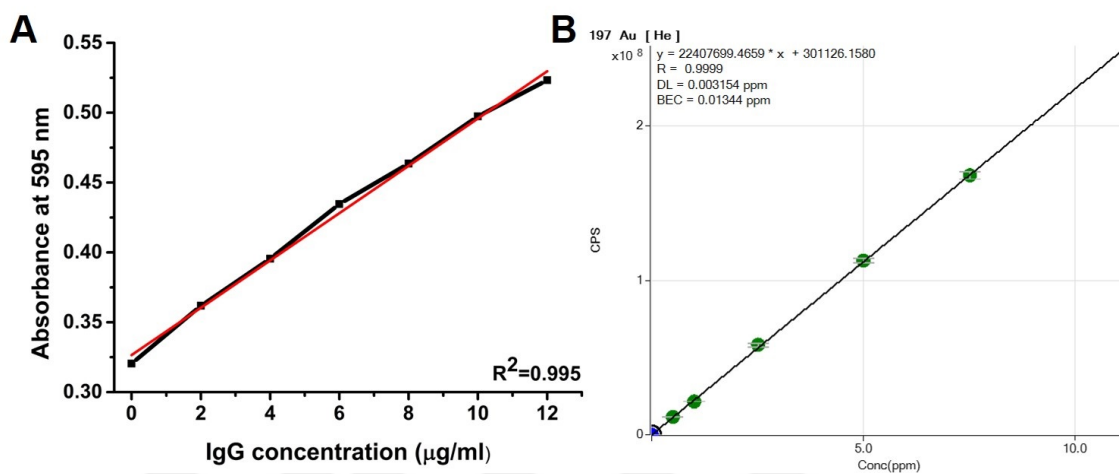


Figure 4.8: Calibration curves of (A) Bradford assay for IgG and (B) ICP-MS for Au quantification.

Table 4.2: Au and IgG concentrations of GNPs.

Sample Names	[Au] (μg/ml)	[IgG] (μg/ml)
GNP	1400	-
Glu-GNP	473	-
GNP-IgG	432	807.4
Glu-GNP-IgG	366	916

laboratory. Rats were divided into seven groups, GNP, IgG, Glu-GNP, GNP-IgG, Glu-GNP-IgG, control-CLP and Sham. Briefly, in day one, CLP model was created as explained in the experimental part and rats were injected with the drug from tail vein immediately after the surgery. GNPs were injected to animals approximately at a dose of 50 μg [Au] per 250g of rats. 24 hours later, rats were killed with guillotine. Spleen, kidney, blood, liver and brain of the rats were collected for ICP analysis. ICP results of four GNP samples are given in the Figure 4.9. Blood analysis indicated that all GNPs were transferred into the organs in 24 h. Blood concentrations were 54.52 ± 21.30 ng/g for GNP but 24.98 ± 5.97 ng/g for Glu-GNP and 162.94 ± 98

ng/g for GNP-IgG. Blood concentration of Glu-GNP-IgG was 74.64 ± 37.52 ng/g. It seems like IgG presence on GNPs increased the blood circulation time may be due to possible interactions of IgG with blood components and even may be with the sepsis-related pathogens. It could have been the larger overall size but larger size usually causes fast blood clearance. On the other hand, glucose conjugation seem to provide a faster blood clearance and uptake by organs. ICP-MS analysis of the brain tissues collected from the test animals showed that all GNPs were able to pass the BBB. On the contrary to the expected, both Glu-GNP (48 ± 9.91 ng/g) and Glu-GNP-IgG (65.29 ± 43.47 ng/g) passed the BBB less than GNP (89.2 ± 18.9 ng/g) and GNP-IgG (115.98511 ± 22.15 ng/g). Glucose may be speeding up organ targeting resulting a fast blood clearance and hence relatively less accumulation in the brain. As expected for nanoparticles, most of GNPs were taken into spleen, liver and kidney. These organs produce different types of macrophages, which are white blood cells of the immune system, to recognize and dispose dangerous and foreign species such as bacteria, viruses, drugs and nanoparticles.

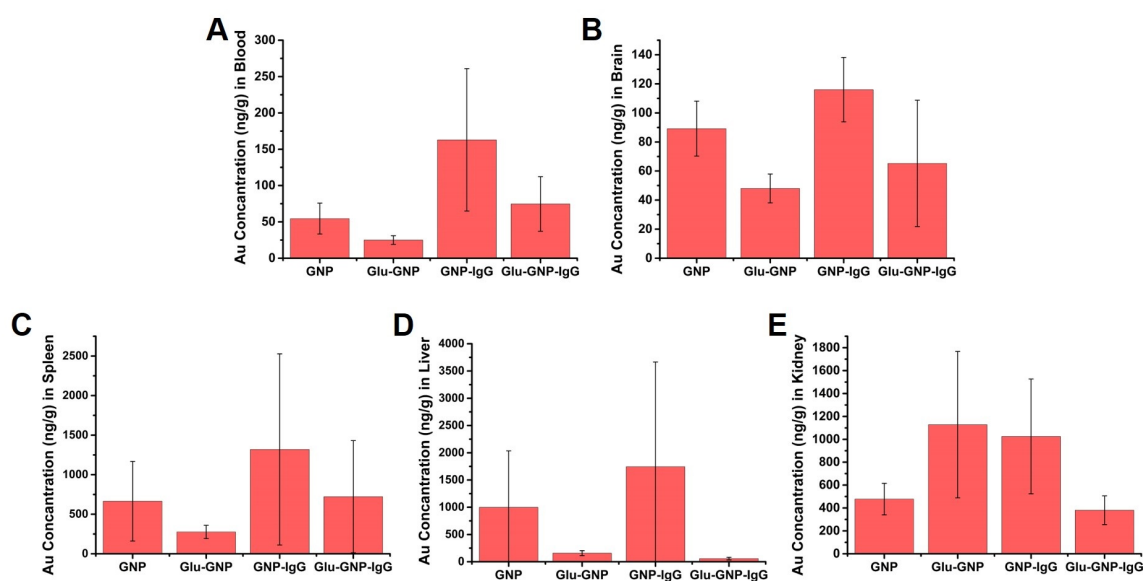


Figure 4.9: Au concentrations in (A) blood, (B) brain, (C) spleen, (D) liver and (E) kidney collected after 24 hours later of injection of GNP, Glu-GNP, GNP-IgG and Glu-GNP-IgG to rats.

4.4 Conclusion

Small, stable, reproducible and biocompatible gold nanoparticles with different surface functionality and composition were synthesized. The main nanoparticle was 3MPA coated gold nanoclusters. Glucose and IgG conjugations to GNPs were achieved in a multiple step synthetic protocol. Reaction conditions and conjugation sequence as well as the molecular weight of PEG linkers were carefully chosen. For the Glu and IgG conjugated GNPs, first Glucose was conjugated to GNPs then the IgG. IgG was oxidized in the Fc fragment to preserve its antigen binding activity on the Fab, and these oxidized carbohydrate units were utilized to conjugate IgG to GNPs via acid-cleavable hydrazone linkages. Immediately after the synthesis, IgG containing GNPs were sterilized under the laminar flow hood to prevent bacterial attack. Au and IgG concentration of nanoparticles were determined via ICP-MS and Bradford assay. Moreover, a successful CLP model was performed on rats with GNP samples and Au concentration in the blood, brain, liver, kidney and spleen were analyzed. Blood analysis indicated that majority of the GNP samples were transferred into the organs in 24 h. As expected, most of GNPs were taken into spleen, liver and kidney. Interestingly, GNP-IgG passed the BBB in higher amount than Glu-GNP-IgG. Therapeutic effect of IgG is still under investigation in Prof. Kaya's laboratory.

Chapter 5

DEVELOPMENT OF NIR-ABSORBING GOLD NANOPARTICLES

5.1 A new approach to synthesize stable, functional, gold-gold sulfide nanoparticles

Synthesis of GGSs are relatively easier than the other NIR absorbing gold nano-species such as gold nanorods and gold nanostars. Yet, reproducible synthesis and stability of GGSs are challenging especially for biomedical applications. In several studies, GGS were coated with capping agents after the GGS formation to increase the stability. Schwartzberg et al. coated GGSs with poly(vinyl pyrrolidone) (PVP) due to relatively inert, water soluble nature.[13] But, they also reported changes in the optical properties of GGS upon PVP addition. Ren et al. used 11-mercaptoundecanoic acid (11-MUA) to stabilize particles and to load cisplatin for both chemo and photothermal therapy.[213] They mentioned the absorbance at NIR region was decreased by 10% after cisplatin loading. Saverot et al. utilized PEG-SH to stabilize GGS after the synthesis.[214] Day et al. stabilized the particles with 2,000 Da ortho-pyridyl-disulfide-poly(ethyleneglycol)-N-hydroxysuccinimide (OPSS-PEG-NHS) linker conjugated with two different antibodies, anti-HER2 and nonspecific anti-IgG.[215] They used antibodies for targeted photothermal therapy and as capping agents and increased the stability of the particles. Additionally, for combined photothermal (PTT) and photodynamic therapy (PDT), Ghorbani et al. used indocyanine green (ICG) as the coating agent and photosensitizer (PS) after the synthesis of GGS.[216] They also mentioned the difficulties of reproducing the GGS and stated that chemists should focus on this problem.

In this study, a simple, reproducible synthesis method for colloiddally stable and functional GGS with NIR absorbance was aimed. GGSs with cationic (25 kDa bPEI), anionic (3MPA) and drug formulated (VCR) capping agents were prepared in one pot synthesis. Their potential in photothermal therapy was also demonstrated. Anionic photosensitizer indocyanine green (ICG) was loaded to bPEI-GGS and a zwitterionic photosensitizer 5-aminolevulinic acid (ALA) was loaded to 3MPA-GGS for the combination therapy that is under investigation.

5.2 Experimental

5.2.1 Materials

Gold (III) chloride solution 30 wt % in dilute HCl (HAuCl_4) and 25 kDa branched polyethylenimine (PEI) was purchased from Aldrich (Germany). Centrifugal filters with 10 and 30 kDa cut-off membranes were purchased from Sartorius. Sodium sulfide (Na_2S) was purchased from Alfa-Aesar. 3-mercaptopropionic acid (3MPA) and sodium borohydrate (NaBH_4) were purchased from Merck. Vincristine sulfate was purchased from Koçak Farma.

5.2.2 Instrumentation

UV-vis absorption spectra were recorded on a Shimadzu UV-3600 spectrophotometer. Dynamic light scattering (DLS) and zeta potential measurements were done using a Malvern Zetasizer Nano-ZS. XPS samples were dropped on silicon wafer and left 2 days in the petri dish for drying. XPS analysis were performed on a Thermo Scientific K-Alpha XPS with Al $K\alpha$ monochromatic radiation (1486.3 eV). Powdered samples were put on a carbon adhesive tape. 50.0 eV pass energy was used for the scans with a flood gun with a 400 mm spot size. A base pressure lower than 9×10^{-9} mbar and experimental pressure of 1×10^{-7} mbar was achieved. The C1s peak at 284.5 eV was used as a reference. High-resolution TEM (HRTEM) imaging was performed using 200 keV electrons in a transmission electron microscope with a spherical aberration-

corrected probe (JEOL JEM-ARM200CF) at SUNUM, by Prof. Cleva Ow-Yang, Istanbul.

5.2.3 Synthesis of bPEI coated GGS

bPEI coated GGSs were synthesized with modification of a reference procedure [13]. 0.1 M Na_2S solution was added on 5×10^{-4} M HAuCl_4 solution and mixed for 3 min at RT to allow Au-S bonding. Next, 1.16×10^{-4} M 25kDa bPEI was mixed with Au-S solution and placed in a preheated (95°C) silicon oil and stirred at a constant speed (300 rpm) for 30 min. A color change was observed from brownish-yellowish color to deep purple as the complete reduction occurs. After cooling down the solution to room temperature, bPEI-GGSs were washed with de-ionized water couple of times using centrifugal filters with 30kDa molecular weight cut-off membrane. Resulting GNP dispersions were stored in a vial covered with an aluminium foil and kept in 4° .

5.2.4 Synthesis of 3MPA coated GGS

5×10^{-3} M 3MPA was added to 5×10^{-4} M HAuCl_4 solution. pH was set to 7.5 and the mixture was stirred for 30 min. Then, 0.1 M Na_2S was added to this mixture and stirred for 90 min at a constant speed (300 rpm). To complete the reduction, freshly prepared 1×10^{-5} M NaBH_4 solution was added at RT. The color change was observed in 2 min after addition of NaBH_4 from colorless to red then deep purple as the reduction takes place. This reaction mixture was mixed over night. 3MPA coated GGS were washed with de-ionized water couple of times using centrifugal filters with 10kDa molecular weight cut-off membrane. Resulting GNP dispersions were stored in a vial covered with an aluminium foil and kept in 4° .

5.2.5 Synthesis of Vincristine coated GGS

VCR coated GGS were synthesized by using the drug purchased from Koçak Farma. It contains mannitol as an additive. For this reaction, all solutions were prepared in an ice bath. In one flask, 4 ml of VCR was mixed with 0.2M NaOH. In another flask,

110⁻³ M HAuCl₄ and 0.2 M Na₂S were mixed for 5 min. Later, VCR-NaOH mixture was poured in Au-S solution and mixed at a constant speed (300 rpm) for 2 hours. VCR-GGSs were washed with de-ionized water couple of times using centrifugal filters with 5kDa molecular weight cut-off membrane. Resulting GNP dispersions were stored in a vial covered with an aluminium foil and kept in 4°C.

5.2.6 Photothermal heating of GGS in solution

To investigate the photothermal potential of three GGS, all samples were filtered with 0.2 μ m filter to get rid of large particles. A 1 ml volume of GGSs were placed in a polystyrene cuvette and irradiated with 795nm diode laser with 800 mW (2.1 W cm⁻²) of laser power for 20 minutes. The temperature increase was followed with a thermocouple.

5.2.7 Isothermal titration calorimetry (ITC) experiments for the determination of photo sensitizer loading on GGSs

ITC experiments were performed on a Nano ITC TA Instruments at 25°C. 1 ml bPEI-GGS (1mg/ml) was added into the cell and 300 μ l ICG (200 μ g/ml) were loaded into the syringe. In a separate experiment, 1ml 3MPA-GGS (140 μ g/ml) was added into the cell and 300 μ l ALA (100 μ g/ml) were loaded into the syringe. GGSs were titrated with 50 portions of 5 μ l PS (ICG and ALA) with 10 min intervals under constant stirring (125 rpm). The heat of reaction after each injection was recorded.

5.3 Results and Discussion

At the initial stage of this work, a published procedure for the synthesis of GGS was repeated to understand the flow of the reaction and the bottlenecks. [13] HAuCl₄ was placed into a preheated water bath. Then Na₂S was added and mixed at a constant speed. The reduction was monitored by UV-vis spectroscopy given in Figure 5.1A. Initially, SPR band did not appeared since the particles were in a cluster form. As the particle growth occurred, SPR band observed at 520 nm. After 180 min, the second

LSPR band in the NIR was not observed as indicated in the reference. Moreover, particles got aggregated and eventually precipitated. The reason of not observing the second LSPR band may be related to the sodium sulfide amount. The reference article just like many others did not specified the Na_2S amount used in the reaction. However, the probable reasons of the second LSPR band was explained by Schwartzberg et al and Mikhlin et al.[13, 55] One of the reasons was the shape inhomogeneity: various shapes such as triangles, plates and rods were present in GGS. The second reason was suggested as possible aggregation during the crystal growth.

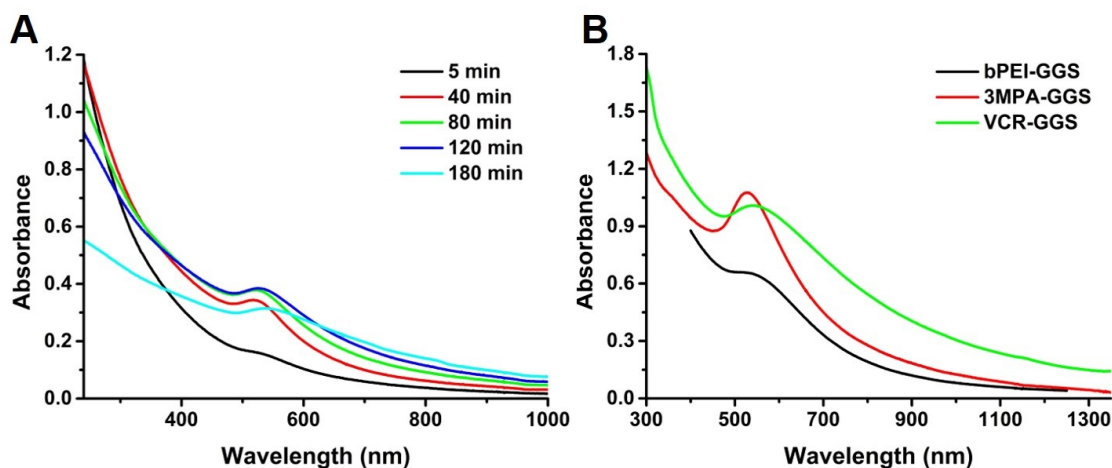


Figure 5.1: UV-vis spectrum of (A) GGS synthesized according to the given reference [13] and (B) bPEI coated GGS, 3MPA coated GGS and VCR coated GGSs synthesized according to the new procedure.

Since GGSs consist of aggregated particles with various shapes, they are stable for only couple of days to couple of weeks. Thus, coating them with a stabilizer is essential for further practical applications. However, addition of coating material was reported to decrease the intensity of the second LSPR band of GGS nanoparticles. Yet, stability of particles increased from couple of days to three months.[56, 13]. To overcome the long-term stability problem, three different GGSs were synthesized with different coating materials in this thesis work. Synthesis of these particles were similar to the GGS adopted from the literature but cationic, anionic or drug formulated capping

agents were added directly during the synthesis of nanoparticles. Figure 5.1B shows the UV-vis spectrum of bPEI, 3MPA and VCR coated GGS. Even though the second LSPR band was not produced, these nanoparticles show absorbance until 1350nm.

Table 5.1 summarizes the size measured with DLS and zeta potential values of these GGSs. bPEI coated GGS were relatively more polydisperse than 3MPA-GGS and VCR-GGS possibly due to the polymeric coating. The intensity based hydrodynamic size of 3MPA-GGS, 122.4 nm, was comparatively larger than bPEI-GGS and VCR-GGS, 60.5, and 70.8 nm. respectively. Probably intermolecular interactions such as hydrogen bonding and electrostatic interactions of carboxylate group of 3MPA increased the intensity based hydrodynamic size. But, the number based hydrodynamic sizes were 15.7 nm for 3MPA and 18.17 nm for bPEI-GGS and 11.7 nm for VCR-GGS.

Table 5.1: Influence of coating material on properties of GGS

Sample name	Size ^a (nm)	Size ^b (nm)	PDI ^c	Zeta Potential (mV)
bPEI-GGS	60.5	18.2	0.36	+20.7
3MPA-GGS	122.4	15.7	0.30	-38.6
VCR-GGS	70.8	11.7	0.30	-42.8

^a Intensity based hydrodynamic size ^b Number average hydrodynamic size and ^c polydispersity index (PDI) calculated from DLS measurement

TEM images (Figure 5.2) exhibited that aggregated gold nanoparticles were produced, which is in agreement with the literature. Core size of GNPs were well correlated with the DLS measurements. SAED pattern generated by TEM proved the crystalline structures of the bPEI-GGS (Figure 5.2A) and 3MPA-GGS (Figure 5.2B). Yet, crystalline structure for VCR-GGS (Figure 5.2C) was not detected. Because, VCR-GGS contains both vincristine drug and mannitol on its surface. Dried mannitol most probably limited the resolution of TEM.

bPEI-GGS, 3MPA-GGS and VCR-GGS were further characterized by XPS (Fig-

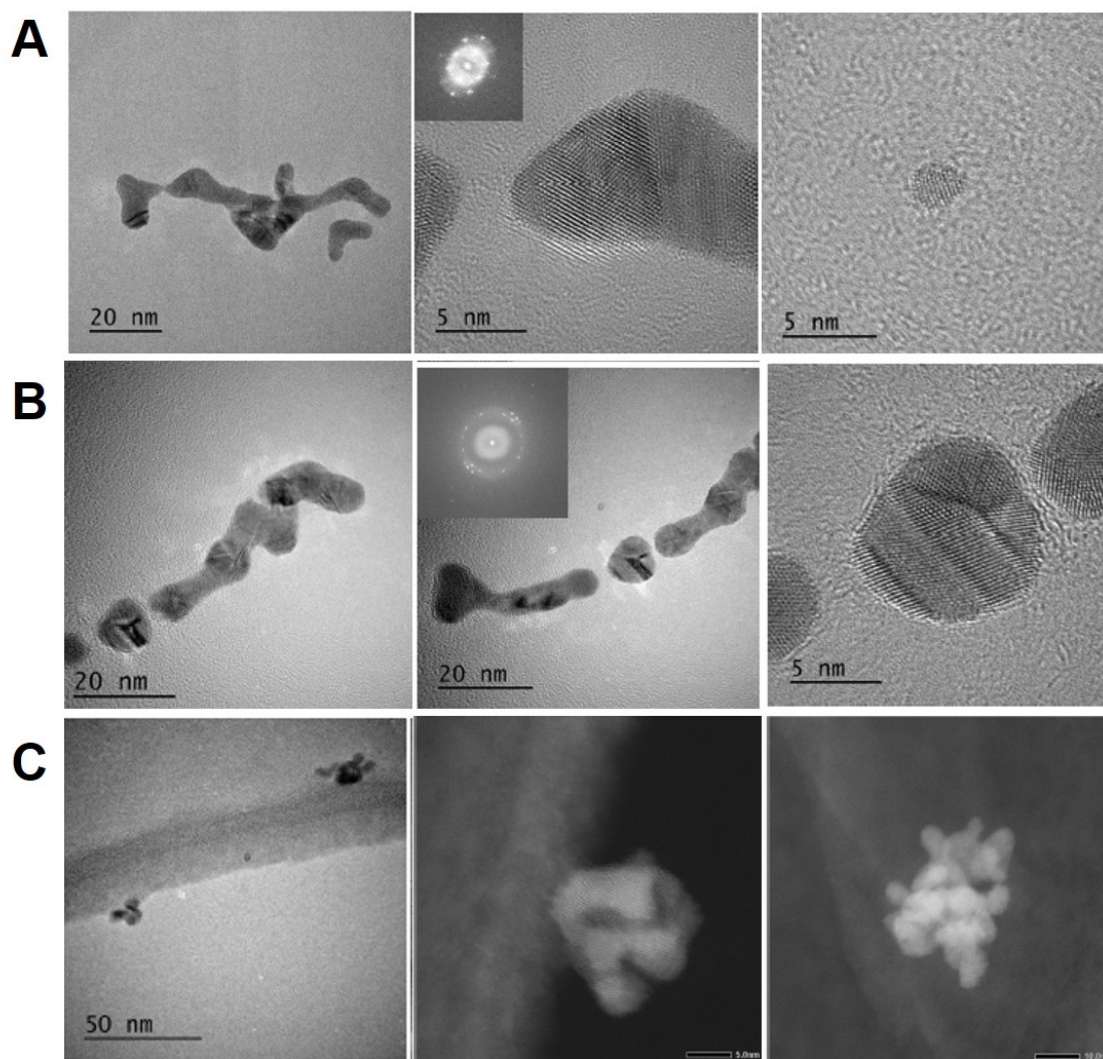


Figure 5.2: TEM images of (A) bPEI-GGS, (B) 3MPA-GGS and (C) VCR-GGS.

ure 5.3). The binding energies (BE) of Au 4 f7 was at ca. 83 eV for all GGS samples which indicates that Au is in its metallic state. The S2p3 peak at ca 161 eV indicates the Au-S bond which was clearly seen in 3MPA-GGS (160.78eV) and VCR-GGS (160.98 eV). Yet, the BE of sulfite appears at ca 167-168 eV. Apparently, the elemental sulfur in bPEI-GGS with the BE at 167.48 eV was completely oxidize during the reaction. Similarly, 3MPA-GGS and VCR-GGS exhibit signals at 167.58 eV. Na₂S stock solution in dH₂O was prepared 3 days before the experiment. Because Schwartzberg et. al. was reported that GGSs produced with aged Na₂S in dH₂O had more intense

2nd LSPR peak than the one produced with fresh Na_2S .^[13] They claimed oxidized sulfur produced more aggregated nanoparticle. Additionally, 3MPA-GGS had third S 2p3 peak at 163.18 eV corresponding to the thiol of the capping agent (3MPA). Three different types of N1s were observed in bPEI-GGS with the BEs 397.23, 398.46 and 399.78 eV. Additionally, a N1s signal was detected at 399.24 eV in a small amount in VCR-GGS. The BEs of N1s at ca 399 eV correspond to bound or oxidized amines and the BE of N1s at ca 397 eV represents to free amines.

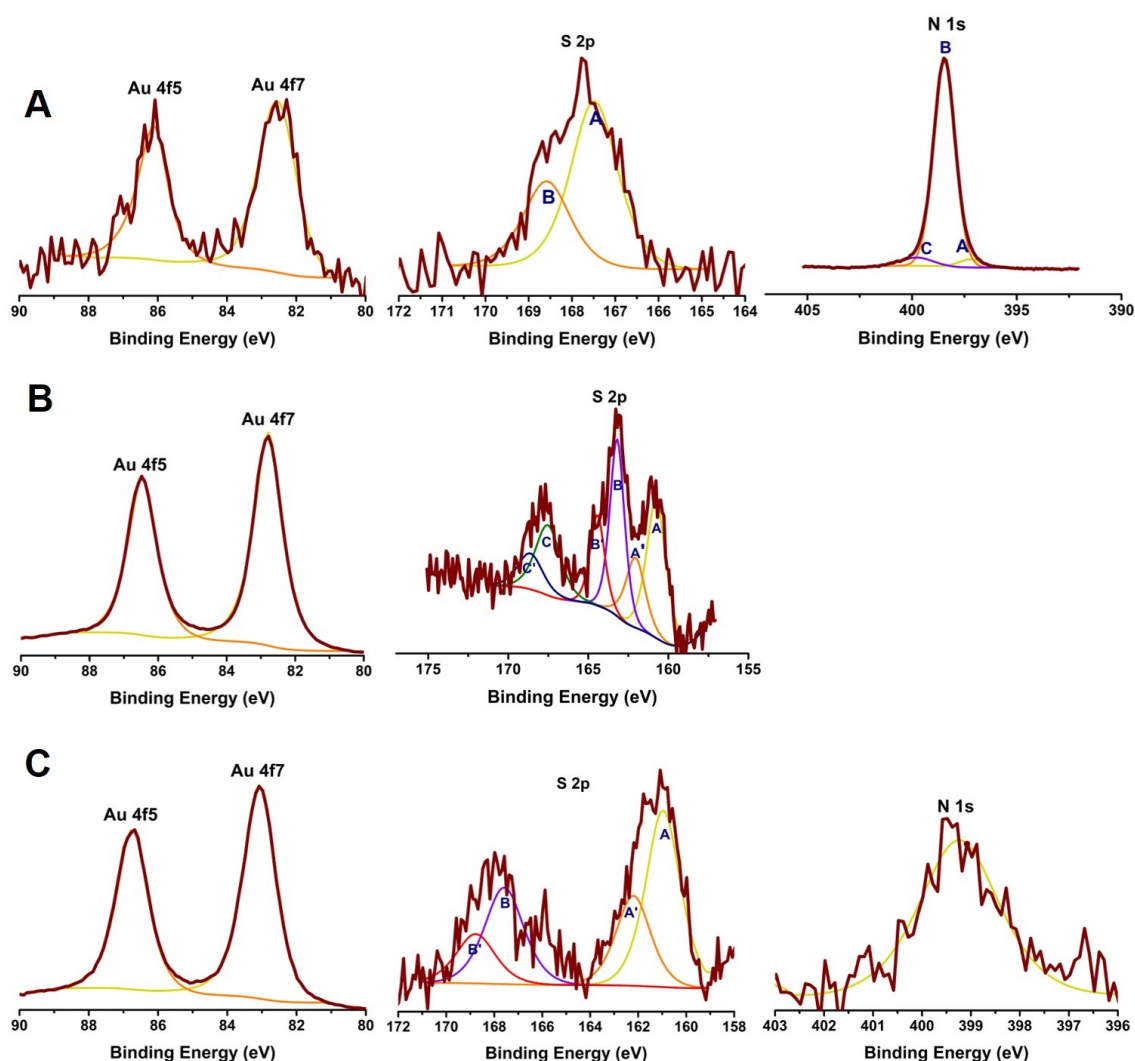


Figure 5.3: XPS spectra of (A) bPEI-GGS, (B) 3MPA-GGS and (C) VCR-GGS.

Furthermore, we have investigated their photothermal potential. bPEI-GGS (75 $\mu\text{g/ml}$ [Au]), 3MPA-GGS (47.4 $\mu\text{g/ml}$ [Au]), VCR-GGS (77 $\mu\text{g/ml}$ [Au]) solutions were irradiated at 795 nm with 800 mW (2.1 W/cm^2) laser power for 20 minutes. The time dependent heating curves and temperature changes (ΔT) are given in Figure 5.4. The absorbance values of bPEI-GGS, 3MPA-GGS and VCR-GGS at 785 nm were 0.197, 0.283 and 0.553 a.u., respectively. Photothermal potential of GNPs depends on the concentration of Au and amount of absorbed light at the certain wavelength. As expected, the highest temperature change (ΔT) observed with VCR-GGS (19 $^{\circ}\text{C}$) due the high Au concentration and strong absorbance at the irradiation wavelength. Even though 3MPA-GGS had higher absorbance intensity than the bPEI-GGS, the Au concentration was higher in bPEI-GGS so that their ΔT was similar, 17 $^{\circ}\text{C}$.

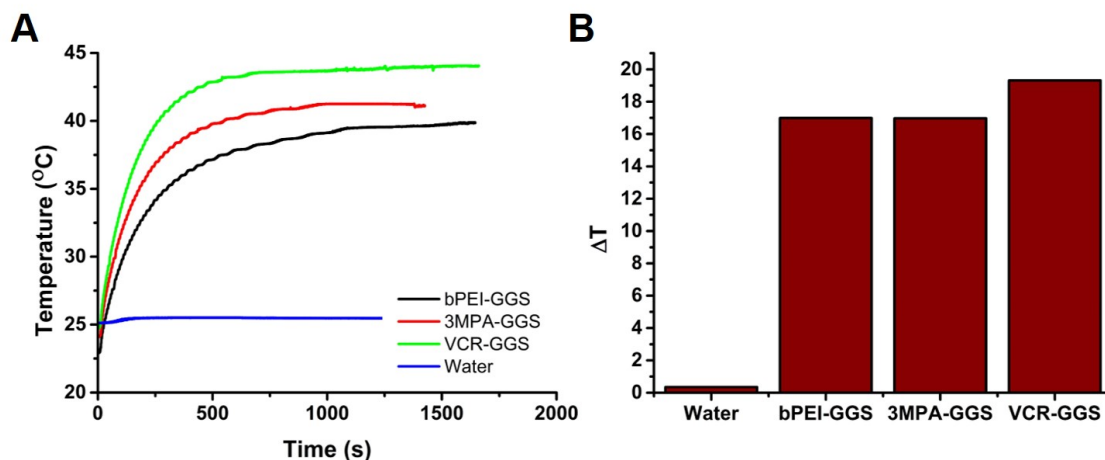


Figure 5.4: (A) The time dependent temperature changes and (B) temperature changes (ΔT) of bPEI-GGS, 3MPA-GGS and VCR-GGS irradiated at 800 mW (2.1 W/cm^2)

In recent years, combination of photothermal (PTT) and photodynamic therapy (PDT) have been studied to create a synergistic effect in cancer treatment.[217] For this purpose, an anionic photo sensitizer (PS), indocyanine green (ICG) was loaded to cationic bPEI-GGS and, a zwitterionic PS, 5-Aminolevulinic acid (ALA) was loaded to anionic 3MPA-GGS. The interactions between the PSs and GGSs and maximum

PS loading capacity was further investigated with isothermal titration calorimetry (ITC). 1 ml bPEI-GGS (1mg/ml) was inserted into the cell and 300 μ l ICG (200 μ g/ml) were loaded into the syringe. In a separate experiment, 1ml 3MPA-GGS (140 μ g/ml) was inserted into the cell and 300 μ l ALA (280 μ g/ml) were loaded into the syringe. GGSs were titrated with 50 portions of 5 μ l PS (ICG and ALA). The decrease in the exotherms of bPEI-GGS after the 23rd injection (23 μ g ICG) indicated that the maximum ICG concentration that could be bound to 1ml bPEI-GGS (1mg/ml) (Figure 5.5A). Thus, the maximum amount of ICG that can be loaded to bPEI-GGS was determined as 2.3%. On the other hand, exotherms of 3MPA-GGS (140 μ g/ml) were remained same after 50 injections of ALA (70 μ g) which indicated a strong and stable electrostatic interaction between 3MPA-GGS and ALA (Figure 5.5B). Therefore, for further PDT applications 50% ALA was loaded on 3MPA-GGS.

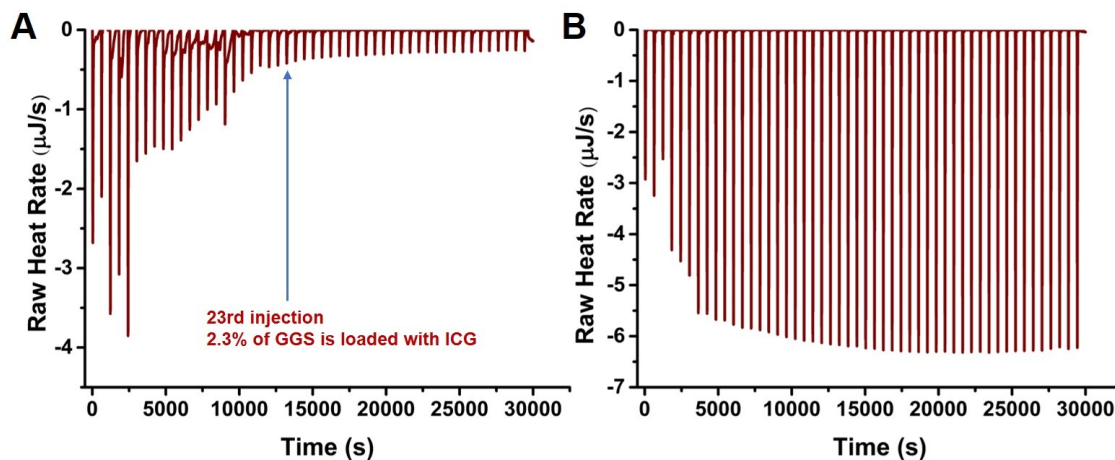


Figure 5.5: Baseline subtracted raw data for the ITC titration of (A) bPEI-GGA with ICG and (B) 3MPA-GGS with ALA.

5.4 Conclusion

Stable, reproducible NIR absorbing gold nanoparticles with cationic, anionic and drug formulated coatings were successfully synthesized in aqueous media. DLS and TEM analysis showed that these particles were consist of lightly aggregated small particles

with a number based hydrodynamic size ca 15 nm. XPS analysis showed that Au was reduced to its metallic state in all samples. Sulfur was found in different states such as oxidized, mono- and polysulfide. Although no sharp second LSPR band was found in the NIR, these particles have a significant absorbance in the NIR that may be utilized for NIR-phototherapy. It was concluded that stabilization of GGS nanoparticles during the crystal growth both reduces the shape and size inhomogeneity seen in the uncoated GGSs with the second LSPR band. Hence, no sharp peak in the LSPR was seen with these GGSs.

However, absorbance of these GGS nanoparticles in the NIR was sufficient to produce photothermal effect. All samples were irradiated with 785 nm laser and the temperature change was up to 19°C which makes them great candidates for PTT applications. Additionally, 2.3% ICG was loaded on bPEI-GGS and 50% ALA was loaded on 3MPA-GGS to investigate the dual therapy potential. Combined therapy potential of bPEI-GGS and 3MPA-GGS for cancer treatment are still under investigation in Dr. Murat Gulsoy's Laboratory (Bogazici University).

Chapter 6

DEVELOPMENT OF SULFONIC ACID FUNCTIONAL SPIONS

6.1 Development of sulfonic acid-functionalized SPIONs for folic acid targeted combined doxorubicin and photothermal treatment of cancer

Super paramagnetic iron oxides (SPIONs) attract researchers from academia to industry due to their unique magnetic properties, surface to volume ratio, biocompatibility and chemical stability.[100] They are widely used in sensors, catalysis, energy applications etc.[218, 219, 220, 221] Moreover,since they are the only metal nanoparticles approved by Food and Drug Administration (FDA), SPIONs have been heavily utilized in biomedical applications such as therapeutics, magnetic hyperthermia, drug delivery, and contrast agents for medical imaging.[222] One of the most preferred synthesis method of SPIONs is the co-precipitation method due to adjustable scale of the reaction, flexibility to provide in-situ coating, and hence provide surface functionality and control size. In co-precipitation synthesis method, ferric (Fe^{+3}) and ferrous (Fe^{+2}) ions with a molar ratio of 1:2 are mixed in de-oxygenated water and treated with a strong base at desired temperature under inert atmosphere. [95] The coating material can be added in-situ or post synthesis.[99] Mostly, carboxylic acid and amine functionalized SPIONs have been used for biomedical applications due to conjugation sites and strong electrostatic interactions with drugs and genes.[223] One interesting functionalization would be with sulfonic acid. Sulfonic acid functionalized SPIONs (SPION-SO_3^-) have been only used in catalysis applications ,to best of our knowledge, such as synthesis of 1-substituted 1H-tetrazoles, Michael addition

reactions, hydrolysis, N-formylation of amines, etc.[100, 109, 224, 225, 226] SPION-SO₃⁻ were synthesized in two different methods. In some studies, first silica coated Fe₃O₄ were produced then reacted with chlorosulfonic acid in dry dichloromethane or chloroform. In other studies, bare Fe₃O₄ was directly reacted with chlorosulfonic acid in dry dichloromethane or chloroform. Interestingly, there are few examples in the literature using sulfonate functionalization of Au, Ag, Pd, Zn and Cd nanoparticles.[227, 228, 229] These particles were developed to mimic heparan sulfate proteoglycans (HSPG) for viral treatment. Yet, there are no SPION based examples.

Recently, photothermal therapy (PTT) potential of SPIONs as well as combination therapies such as chemo - PTT and magnetic hyperthermia - PTT have attracted a great attention. For chemotherapy, drugs can be loaded to the coating of the SPIONs to increase the cell internalization, biocompatibility and solubility of drugs. Doxorubicin is a chemotherapy drug to treat a large number of cancers, such as leukaemia, ovarian cancers and particularly last stage breast cancers. [230] Folic acid is a popular targeting ligand due to the high binding affinity to folate receptors which is overexpressed in many tumor types, especially in ovarian cancer.

In this study, one-pot, aqueous synthesis of sulfonic acid functionalized SPIONs by using 3-(trihydroxysilyl)-1-propanesulfonic acid (THSPSA) as a coating material have been developed which is quite unique in the literature, especially for biomedical applications. Additionally, PTT potential of SPION-SO₃⁻ in solution was investigated under 795 nm laser irradiation. For combined PTT and chemotherapy, DOX was successfully loaded to the nanoparticles. Moreover, for targeting an ovarian cancer cell line, Hela, and the breast cancer cell line MDA-MB 231, SPION-SO₃⁻ was also electrostatically loaded with folic acid (FA).

6.2 Materials and Methods

6.2.1 Materials

Iron(II) chloride and iron(III) chloride were purchased from Merck (purity levels 99%, Germany). 3- (trihydroxysilyl)-1-propanesulfonic acid, (30-35% in water) was obtained from Gelest (USA). Ammonium hydroxide solution (25-30%) was purchased from Sigma-Aldrich (Germany). Vivaspin 20 centrifugal filters (10 kDa MW cut-off) were obtained from Sartorius (Germany). Folic acid was purchased from Sigma-Aldrich (Germany). Doxorubicin-HCl was purchased from GoldBio (USA).

6.2.2 Synthesis of SPION-SO₃⁻ Nanoparticles

Sulfonic acid functionalized SPIONs (SPION-SO₃⁻) were synthesized by mixing FeCl₃6H₂O, FeCl₂4H₂O and 3-(Trihydroxysilyl)-1-propanesulfonic acid (THSPSA) at a mole ratio of Fe⁺³/Fe⁺² of 2/1 and 2.36 ml THSPSA. Briefly, 0.256 g Fe⁺² and 0.69 g Fe⁺³ were mixed in de-oxygenated DI water under nitrogen gas until the temperature raised from room temperature (RT) to 85°C. Then, 4 mL 4.5 M NH₄OH and 2.36 ml THSPSA was injected at the same time under vigorous stirring. The solution was mixed for 1 hour at that 85°C and cooled to RT. The final sample was left on a magnet overnight to remove any precipitated particles. Finally, SPION-SO₃⁻s were washed several times with DI water using Vivaspin 20 centrifugal filters (10 kDa MW cutoff) and stored at room temperature.

6.2.3 Isothermal titration calorimetry (ITC) experiments for the determination of Doxorubicin loading on SPION-SO₃⁻

ITC experiments were performed on a Nano ITC TA Instruments at 25°C. 1 ml 1mg/ml SPION-SO₃⁻ was inserted into the cell and 300 µl 2 mg/ml ICG were loaded into the syringe. SPION-SO₃⁻ were titrated with 25 portions of 10 µl DOX with 10 min intervals under constant stirring (125 rpm). The heat of reaction after each injection was recorded.

6.2.4 Doxorubicin and folic acid loading on SPION-SO₃⁻

The loading concentration of DOX was determined by ITC experiment. To load 10% DOX with respect to the organic coating amount of NPs, 8 ml 4.13 mg DOX was added dropwise to 10 mL SPION-SO₃⁻ (5 mg/mL) solution and mixed at 750 rpm for 2 h at room temperature. Then 10 ml of this solution was separated and 1.9110⁻⁶ mole folic acid was added and mixed 2 hours at RT. Because the loading amounts were determined by ITC, samples were directly filtered with 0.2 μm CA steril filter under the laminar flow hood. The drug loading amount was calculated from standard calibration curve prepared from the absorbance of free DOX at 487 nm. Drug encapsulation efficiency (EE) and loading efficiency (LE) were calculated by using following formulas: EE (%) = [Weight of loaded drug/Weight of drug input] x 100 LE (%) = [Weight of loaded drug/Weight of drug loaded SPIONs] x 100

6.2.5 Instrumentation

UV-vis absorption spectra were recorded on a Shimadzu UV-3600 spectrophotometer. Dynamic light scattering (DLS) and zeta potential measurements were done using a Malvern Zetasizer Nano-ZS. Fluorescence spectra was recorded on a Cary Eclipse Fluorescence Spectrophotometer. Thermal gravimetric analysis (TGA) was performed by using TA Q500 under nitrogen atmosphere. The dried sample was heated from 2°C to 100°C with 10°C/min rate, stayed 30 min at 100°C in order to vaporize the humidity in dried sample. Then, it was heated from 100°C to 660°C with 10°C/min rate. Inductively coupled plasma mass spectroscopy (ICP-MS) was performed by using Agilent 7700x ICP-MS. Magnetic characterization was performed on a Vibrating Sample Magnetometry System (QuantumDesign PPMS9T) at 305 K. Functional groups were investigated using the dried samples and Thermo Scientific Nicolet iS10 ATR-IR.

6.3 Results and Discussions

6.3.1 Synthesis and Characterization of SPION-SO₃⁻ Nanoparticles

Sulfonic acid functionalized SPIONs (SPION-SO₃⁻) were prepared via a simple coprecipitation method. SPION-SO₃⁻ exhibit strong absorption in UV-vis region due to its black-brown color with magnetization between -20 and +20 emu/g at 305 K (Figure 6.1(A-B)).

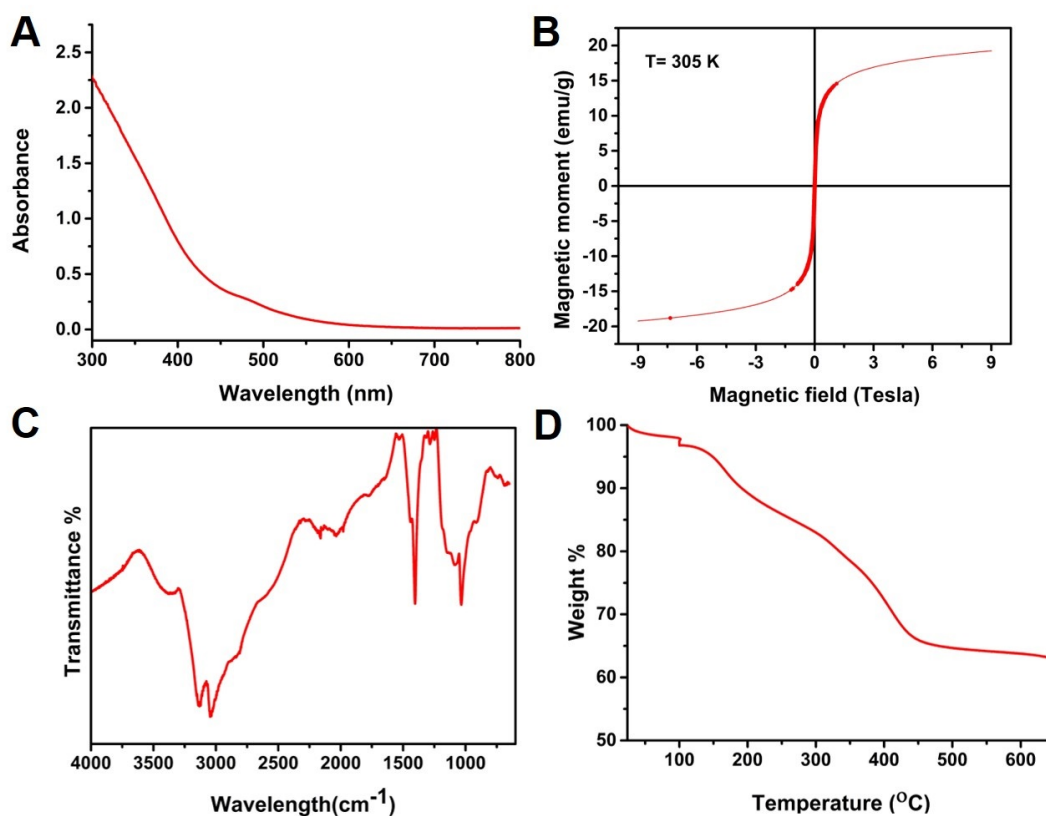


Figure 6.1: (A) UV-vis spectrum, (B) magnetization curve at 305K, (C) FTIR spectrum and (D) thermogram of Sulfonic acid functionalized SPION

Functional groups of sulfonic acid functionalized SPIONs were analyzed by FTIR (Figure 6.1C). The broad peak at 3400-3200 cm⁻¹ is stretching vibrations of -OH from either unbound silanol group or physically absorbed water. The broad peak between 1220 and 830 cm⁻¹ is the finger print region of Si-O-Si, Si-OH and Si-O.[231, 232, 233]

The peak at 1035 cm^{-1} was assigned to asymmetric stretching vibrations of Si-O-Si, the shoulder at 956 cm^{-1} was attributed to Si-OH stretching vibrations. The peak at 1417 cm^{-1} was associated with the stretching vibrations of S=O from sulfonic acid groups.[231, 234] The peak at 582 cm^{-1} is the characteristic Fe-O band of Fe_3O_4 . [235] The organic content of SPION-SO_3^- was calculated 35% from the thermogram given in the Figure 6.1D.

TEM images of SPION-SO_3^- (Figure 6.2) indicate that SPION-SO_3^- are spherical with an average crystal size of $4\text{ nm} \pm 3\text{ nm}$. Figure 6.2B shows the EDX mapping images of SPION-SO_3^- . The EDX mapping clearly determined that the core of the particle consisted of Fe and O coated with silicon.

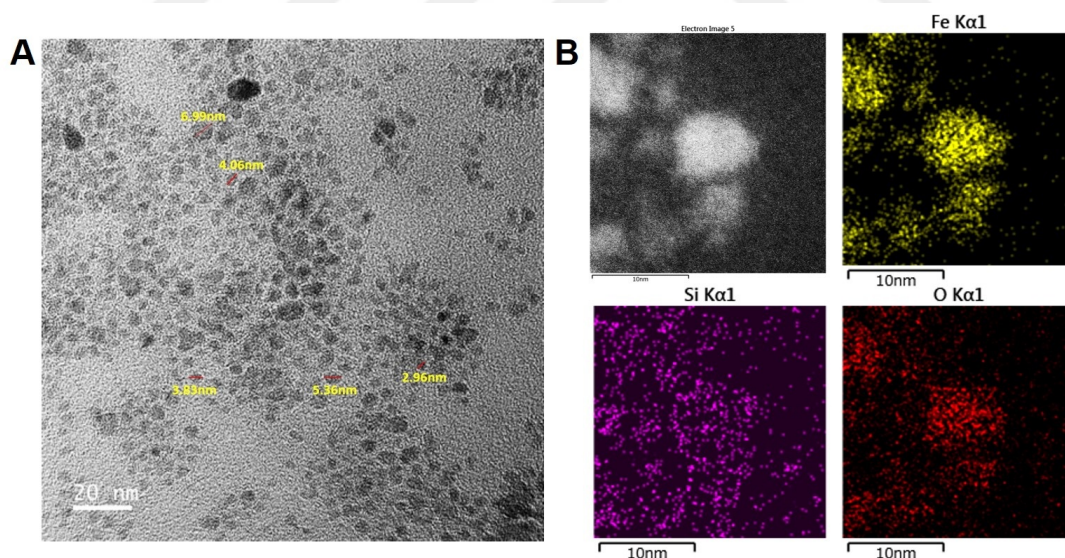


Figure 6.2: (A) TEM image of SPION-SO_3^- , (B) TEM image of SPION-SO_3^- to be analysed for EDX mapping, map of Fe, Si, O concentrations.

Furthermore, the hydrodynamic size of the particles were characterized by dynamic light scattering (DLS) (Table 6.1). Intensity and numbered based hydrodynamic size of SPION-SO_3^- were ca 78.8 nm and 37.84 nm ,respectively, with a -60.0 eV zeta potential.

Table 6.1: Hydrodynamic size and zeta potential values of SPION-SO₃⁻, DOX-SPION-SO₃⁻ and DOX-SPION-SO₃⁻-FA

Sample name	Size ^a (nm)	Size ^b (nm)	PDI ^c	Zeta Potential (mV)
SPION-SO ₃ ⁻	78.8	37.84	0.44	-60.0
DOX-SPION-SO ₃ ⁻	97.5	34.6	0.24	-44.7
DOX-SPION-SO ₃ ⁻ -FA	112.0	28.4	0.23	-41.5

^a Intensity based hydrodynamic size ^b Number average hydrodynamic size and ^c polydispersity index (PDI) calculated from DLS measurement

6.3.2 Investigation of PTT potential of SPION-SO₃⁻ Nanoparticles

The photothermal potential of SPION-SO₃⁻ solution was investigated under 795 nm laser irradiation as a function of nanoparticle concentration and laser power (Figure 6.3). Firstly, 1, 5, 10 and 20 mg/ml SPION-SO₃⁻ was irradiated with 795 nm at 2.1 W/cm² laser power for 20 min. Even 1 mg/ml SPION-SO₃⁻ provided 8°C temperature increase (ΔT) which is quite attractive for mild hyperthermia. As the concentration increased, temperature increase was increased up to 25.8°C with 20mg/ml SPION-SO₃⁻ which is quite unique in the literature. 20 mg/ml SPION-SO₃⁻ solution was also irradiated with 0.8 and 1.3 W/cm² laser power. ΔT was 11°C at 0.8 cm² and 18.3°C at 1.3 W/cm² laser power. Lastly, stability of photothermal response was studied with on and off laser cycles. Same ΔT was observed after each cycle indicating stability and possibility of using the same nanoparticle over and over again (Figure 6.3C).

6.3.3 Nanoparticles for Combination Therapy

For combined targeted chemo- and photothermal therapy, doxorubicin (DOX) and folic acid (FA) was electrostaticly loaded to SPION-SO₃⁻. The loading capacity of the SPION-SO₃⁻ was firstly studied with isothermal titration calorimetry (Figure 6.4A). Briefly, 1mg/ml SPION-SO₃⁻ was titrated with 2 mg/ml DOX. Strong exotherms was

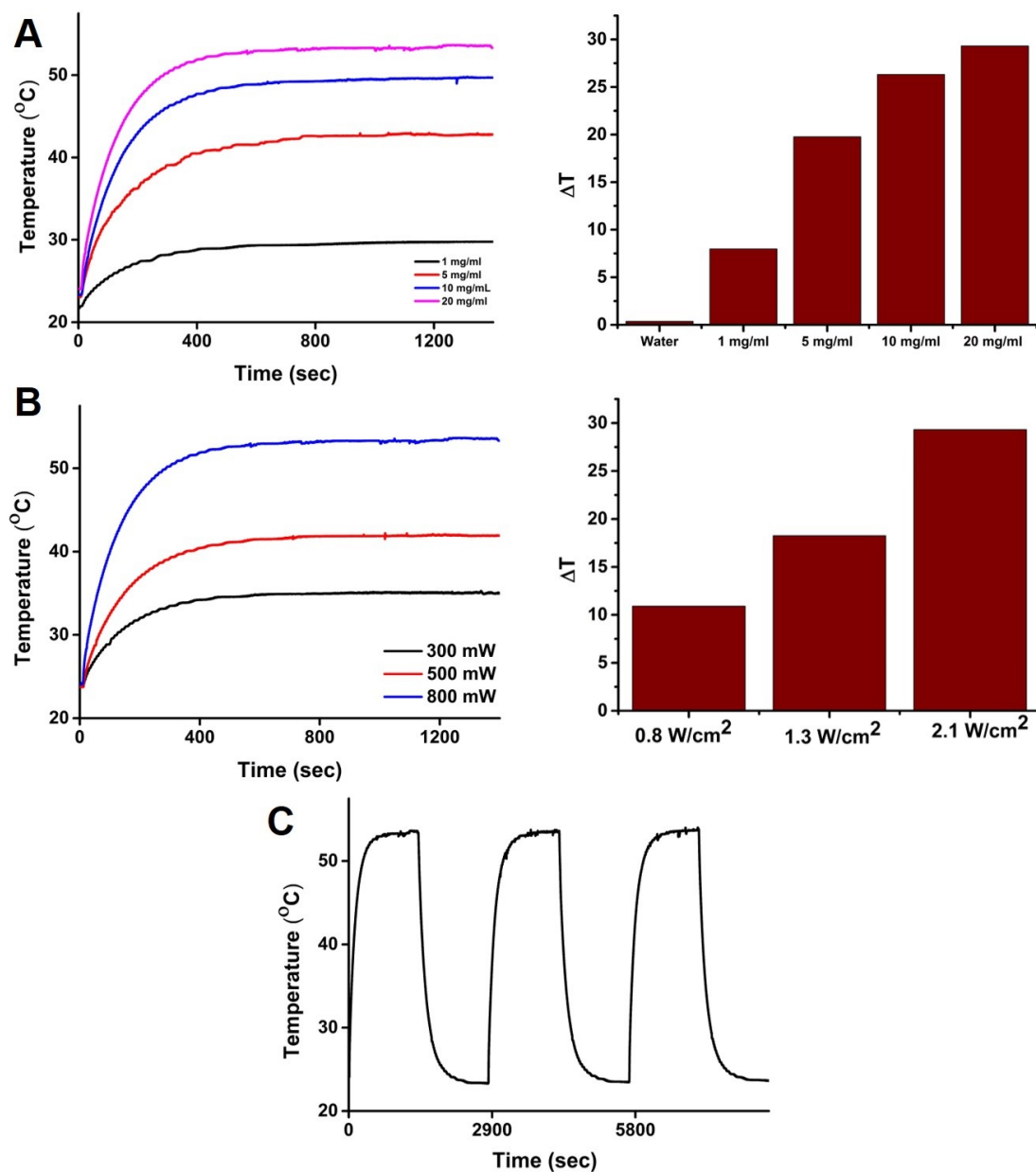


Figure 6.3: Time dependent change in temperature of SPION-SO₃⁻s (A) at different SPION-SO₃⁻ concentrations) irradiated at 2.1 W/cm² (B) irradiated at different intensities 0.8W/cm² (300mW), 1.3W/cm² (500mW) and 2.1W/cm² (800mW) (20 mg/ml SPION-SO₃⁻ solution), and (C) after laser on/off cycles (20 mg/ml) at 2.1 W/cm².

observed at the beginning of the titration, and exponentially decreased. Yet, after 13th addition (%26 percent of overall NP concentration) endotherms were observed.

Probably, SPION-SO₃⁻ was saturated with DOX and dilution of the solution or the precipitation of the particles created the endotherms. According to the exotherms, DOX in the amount of 10 wt% with respect to the coating of SPION-SO₃⁻ were successfully loaded. However, when test samples for in vitro experiments were prepared part of the DOX was stuck on the sterile filter. Yet, the Fe concentration was remained similar. UV-vis spectroscopy (Figure 6.4B) and ICP (Table 6.1) analysis confirmed that Fe concentration remained similar while DOX concentration was decreased.

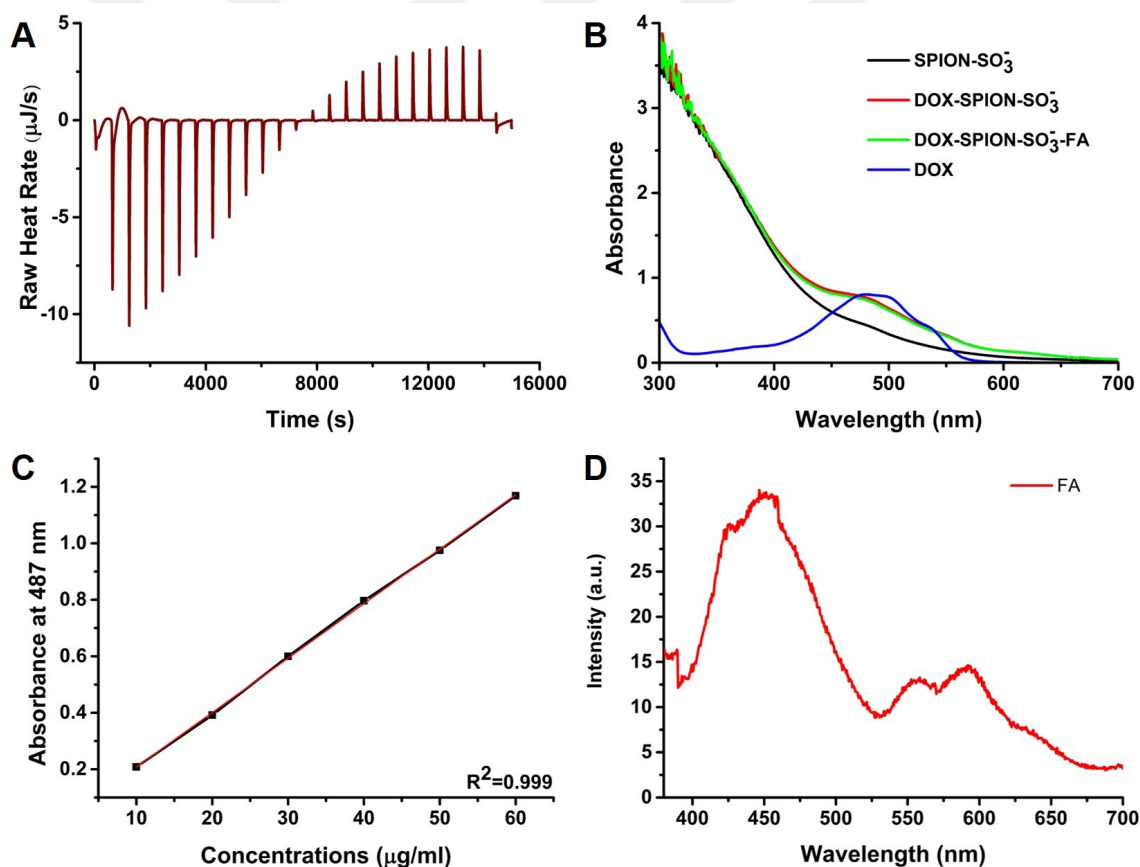


Figure 6.4: (A) Baseline subtracted raw data for the ITC titration of (A) SPION-SO₃⁻ with DOX. (B) UV-vis spectra of DOX and FA loaded SPION-SO₃⁻. (C) UV calibration curve of DOX. (D) Fluorescence spectrum of FA excited at 375 nm.

Therefore, the DOX concentration was calculated again after filtration by using the UV calibration curve of free DOX at 487 nm with subtracting absorbance of SPION-SO₃⁻ given in the Figure 6.4C. EE and LE were determined as 69.4% and 6.9 mol% for

DOX-SPION-SO₃⁻ and 63% and 6.32 mol% for DOX-SPION-SO₃⁻-FA, respectively. Fe, DOX and loaded FA concentrations were summarized in the Table 6.2. Folic acid has an absorbance at 377 nm. Therefore, bound FA amount from the SPION solution cannot be measured from the absorbance spectrum of the sample. [236] Yet, the presence of folic acid was detected with fluorescence spectroscopy given in the Figure 6.4D. Additionally, hydrodynamic size and zeta potential analysis confirmed the electrostatic loading of FA (Table 6.1). The hydrodynamic size of NPs were increased from about 28 to 38 nm while zeta potential was reduced from -60 to 41.5 eV after DOX and FA loading, which is in line with expectations since anionic groups are consumed by these cargo molecules.

Table 6.2: Concentrations of Fe⁺³, DOX and FA in SPION-SO₃⁻, DOX-SPION-SO₃⁻ and DOX-SPION-SO₃⁻-FA

Sample name	Fe ⁺³ (mg/ml)	DOX (μg/ml)	FA (μg/ml)
SPION-SO ₃ ⁻	0.76	-	-
DOX-SPION-SO ₃ ⁻	0.74	159.64	-
DOX-SPION-SO ₃ ⁻ -FA	0.74	146	84.1

6.4 Conclusion

One-pot, anionic and stable sulfonic acid functionalized SPIONs were successfully synthesized and characterized. These particles had about 4 nm crystalline core and ca 37 nm hydrodynamic size which is well within the desired ultra-small size regime. SPION-SO₃⁻ exhibited a dose and laser intensity dependent temperature increase under 795 nm irradiation with acceptable laser powers. Additionally, due to its anionic nature, the chemotherapy drug DOX and targeting molecule FA was successfully loaded to these nanoparticles. Targeted chemo- and photothermal therapy potential of SPION-SO₃⁻ is still under investigation by Dr. Ceyda Açılan Ayhan and her team.

Chapter 7

CONCLUSION

Understanding the behavior of nanoparticles and deciding on the appropriate surface functionality are crucial for nanoparticle-based applications. Nanoparticles tend to aggregate easily with a small change in their surrounding environment, such as pH change or salt addition. It is important to produce nanoparticles with long-term stability and similar properties in each batch of synthesis, especially for biomedical applications. In this thesis, different synthetic methods and coating materials were investigated to determine their influence on the particle size, stability, and optical properties for variety of applications such as gene and drug delivery, photothermal therapy and catalysis. A broad portfolio of gold nanoparticles with cationic or anionic nature and absorbance in either visible or NIR region was produced by changing the coating and reducing agents.

In the second chapter, small, cationic GNPs, which are stable over the years, were produced by the direct reduction of auric acid with bPEI (0.6-25kDa) in water at a mild temperature. In controlled studies wherein only, a single variable was changed, it was found out that basic pH is favored for the synthesis since bPEI provides better reduction and surface binding at basic pH values. This was the first report on the importance of reaction pH in PEI based reduction of auric acid. Additionally, the MW of bPEI did not influence the core size of GNPs as much as it influences the hydrodynamic size, hence the agglomeration. Therefore, low MW PEI was able to produce small and stable nanoparticles at correct stoichiometry, again for the first time in the literature. The partial oxidation of bPEI during the reduction of auric acid or its binding to GNP surface did not hamper the gene binding and delivery potential of the polymer. Indeed, it (PEI25-Au) showed better transfection efficiency

than commercial transfection agent, FuGENE HD. Although PEI1.8-Au did not show an appreciable transfection efficiency, as expected from a low MW PEI, such small cationic GNPs may be used in alternative applications such as drug delivery, targeted therapy, and catalysis. Results of this work was published in New Journal of Chemistry. A TUBITAK 1001 project has been accepted to use these GNPs to deliver genes across the BBB by using bPEI-GNPs in collaboration with Prof. Mehmet Kaya and Prof. Bülent Ahışah.

In the third chapter, the similar synthesis approach was adopted to GNPs produced by direct reduction of auric acid with linear PEI (lPEI) using two slightly different synthetic protocols: one in which lPEI is dissolved by heating, and the other by acidification. The influence of pH on the particle formation and stability was studied between pH 3.5 and 10 for the first time for lPEI-GNP. This study revealed that protonation of some amine groups is necessary for colloidal stability in case of lPEI, and it does not prevent the reduction of auric acid to GNPs. Colloidally stable gold nanoparticles in small hydrodynamic sizes with low molecular weight lPEI (2.5 kDa), as well as 25kDa lPEI were synthesized successfully. This was a task claimed impossible in the literature. MW did not influence the hydrodynamic size, stability or particle shape, but low MW lPEI provided faceted particles. Overall, dissolving lPEI by acidification, using lPEI/Au ratio of 1/21 was determined as the best approach and careful centrifugal precipitation procedure as a safe cleaning protocol to produce small and stable, colloidal lPEI/GNPs. For storage purposes, we also suggest acidic pH. These GNPs were also demonstrated as an efficient catalyst for degradation of both anionic and cationic azo dyes which is quite unique in the literature, presenting a potential as a universal catalyst. Electrostatic attraction, as well as cation- π and cation-lone pair electron interactions, are suggested as the reason behind such universal behavior. This is indeed a remarkable finding that eliminates the need for a new catalyst for different dyes. Results of this work are submitted to Langmuir for publication.

In the fourth chapter, small, stable anionic gold nanoparticles were synthesized

with the reduction of auric acid in water by using 3MPA as the coating and NaBH_4 as the mild reducing agent. The core size and the hydrodynamic size of the GNPs were ca 2 nm and 12 nm, respectively, indicating the formation of ultrasmall GNPs. Those nanoparticles were primarily prepared as a biocompatible nanocarrier for enhanced transport of polyclonal IgG across the blood-brain barrier (BBB) in sepsis treatment. Therefore, to transport these particles across the BBB more efficiently, GNPs were decorated with glucose via a PEG spacer to allow easy recognition and IgG was conjugated from its Fc fragment with acid cleavable hydrazone linkages to preserve its antigen-binding activity. This required a tedious work, appropriate ordering of the conjugations, and finding the best operation and storage conditions. IgG is very prone for a bacterial attack; hence sterilization is crucial but still, IgG-GNPs cannot be stored for a very long time under laboratory conditions. These GNPs were prepared in ca 13 nm hydrodynamic size. The concentration of IgG on the surface of GNPs was determined with Bradford assay and Au concentration was determined with ICP for appropriate dosing in animal studies. A successful CLP model was created by Prof. Mehmet Kaya and his team in accordance with ethical rules on albino rats. They have created CLP model on day one and injected the prepared drugs (GNP, Glu-GNP, GNP-IgG, Glu-GNP-IgG) from tail vein immediately. 24 hours later rats were killed with the guillotine. According to the Au concentration of the blood, spleen, kidney, liver, and brain of the rats determined by ICP, all GNPs were transferred into the organs. As expected, most of GNPs were taken into spleen, liver, and kidney. Interestingly, GNP-IgG passed the BBB in the greatest amount. The therapeutic outcome of IgG is still under investigation in Prof. Kaya's laboratory.

In the fifth chapter, a new approach for producing stable, NIR absorbing gold nanoparticles were developed with cationic, anionic and drug formulated coating in aqueous media. These particles are relatively new, gold-gold sulfide nanostructures with functional, stabilizing coatings. XPS analysis showed that Au was reduced to its metallic state in all samples and sulfur was found in different oxidation states such as oxidized, mono- and polysulfide forms. These particles were prepared in ca

15 nm hydrodynamic sizes and with excellent colloidal stability, which lacked in all reported versions in the literature. The LSPR band of these particles appeared at ca 530 nm with a long absorbance tail up to 1350 nm. In the literature, some of the examples of GGS nanoparticles seem to have their LSPR band in the NIR, but they are a collection of mostly aggregated nanoparticles with various shapes and sizes and are produced without a coating. DLS and TEM analysis showed that GGS prepared here are composed of small and sometimes aggregated particles. In situ coating of the growing crystal should be narrowing down the size and shape distribution as well as preventing excessive aggregation, therefore, providing colloidally stable GGS but not as a strong absorbance in the NIR compared to bare GGS reported in the literature. However, in the PTT experiments performed with these particles, it was concluded that they absorb enough in the NIR to provide effective temperature increase (19°C) upon 20 min irradiation at 785 nm (2.1 W cm^{-2}). These particles were further loaded with photosensitizers to test their potential for the combination of PTT and PDT against cancer. Specifically, 2.3% ICG was loaded electrostatically to bPEI-GGS which would provide image-guided PTT+PDT and 50% ALA was loaded electrostatically to 3MPA-GGS and their therapeutic efficiency is being an investigation by Dr. Murat Gulsoy's Laboratory (Bogazici University). A new project has been submitted to TUBITAK for photoacoustic imaging by using unique optical properties of these new NIR-Absorbing GNPs, as well as the other structures produced in this thesis work by Assoc. Prof. Burin Ünlü (Bogazici University).

In the last chapter, a new synthesis method for sulfonic acid-coated SPION was developed in aqueous media, utilizing the experience developed in nanoparticle stabilization during GNPs and with the motivation of phototherapy work initiated by GGS nanoparticles produced in this thesis work and SPION-based PTT studies that are currently being conducted in our laboratory. SPION-SO_3^- was synthesized with the co-precipitation method and in-situ coating with (trihydroxysilyl)-1-propanesulfonic acid. The hydrodynamic size and the core size of these SPIONs were 37 nm and 4nm, respectively. SPION-SO_3^- exhibited a dose and laser intensity-dependent tempera-

ture increase when excited with a 795nm diode laser. Additionally, due to its anionic nature, the chemotherapy drug DOX and the targeting molecule FA was successfully loaded to these SPIONs electrostatically. Targeted chemo- and photothermal therapy potential of SPION-SO₃⁻ is still under investigation by the group of Dr.Ceyda Açılan Ayhan. A new Horizon 2020 project (ID: SEP-210588049) was submitted with a broad team of European researchers to utilize these nanoparticles as well as GNPs produced in this thesis work in detection and therapy of RA.

Overall, in this thesis, it was shown that stable and functional GNPs with different surface chemistry and with absorbance in the UV-Vis or UV-Vis-NIR can be synthesized in a simple, economic and reproducible way which was one of the main objectives of this thesis work. Here, PEI is used as a unique polyelectrolyte and its pH-dependent protonation and lone pair electrons were utilized in the reduction of auric acid and stabilization of GNPs as well as much less known GGS nanoparticles. A unique effort towards understanding the nature of GGS nanoparticles resulted in the formation of stable GGS with effective absorbance in NIR region which is crucial for photocatalysis and photothermal therapies wherein safe laser wavelengths (NIR) are necessary as the next step in the technology. A difficult task, carrying IgG across BBB with GNPs were attempted and initial proof of success is provided here. Experience gained in controlling properties of GNPs and involvement in PTT initiated the SPION study where sulfonic acid functional SPIONs with an objective of imaged guided photo/chemo/gene therapies can be investigated with. A simple and green synthetic route was shown to produce small SPIONs with great PTT potential, here. As was indicated at appropriate places, the main goal of this thesis was controlled synthesis of such novel particles and their use in variety of applications are partially done by proof of principle studies by Özge Çavuşlar and more is being done and will be done by the collaborators in different fields.

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