ASSEMBLY OF PEPTIDE COATED GOLD NANOPARTICLES

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ABSTRACT

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The assembly of nanoparticles (NPs) into the desired organizations and patterns is critically important for construction of higher structures using nanoparticles as building blocks. The future of nanotechnology mostly depends on the directing the NPs to desired locations and orientations in the construction of predetermined structures. Peptide modification of NPs has been increasingly used for this purpose. In this regard, we designed different sized and charged peptides that can stably bind to gold nanoparticles (AuNPs). Then, we have investigated how the peptide binding to the nanoparticle surface can influence the assembly of the AuNPs in suspension and on hydrophilic surfaces from a drying droplet of their colloidal suspension. We first studied the effect of anionic peptide modification on assembly of the AuNPs in suspension and on surfaces and then we studied the effect of cationic peptide modification. We found that the peptide charge along with the size has a dominating effect on the behavior of the AuNP in suspension and on surfaces. The knowledge acquired from this work may offer simple and new techniques in assembly of nanostructures.

ÖZET

PEPTİD KAPLI ALTIN NANOPARÇACIKLARIN DÜZENLENMESİ

Nanoparçacıkları yapı taşı olarak kullanarak daha gelişmiş yapıların derlenmesinde kendiliğinden düzenlenmeyi kullanmak çok önemlidir. Nanoteknolojinin geleceği de büyük oranda nanoparçacıkların planlanan şekilde düzenlenerek istenen yapıların oluşturulmasına bağlıdır. Bu amaçla nanoparçacıkların peptidlerle işlevselleştirilmesi gittikçe artmaktadır. Biz de bu amaçla farklı yük ve uzunlukta ve altın nanoparçacık yüzeyine bağlanabilen peptidler tasarladık. Sonrasında, yüzeye peptide bağlanmasının süspansiyon içerisinde ve damlatılan hidrofilik yüzeyde süspansiyonun kurumasıyla altın nanoparçacık düzenlenmesine etkisini araştırdık. Önce negatif yüklü peptidlerle modifiye edilmiş nanoparçacıkların sonrada pozitif yüklü peptiddlerle modifiye edilmiş altın nanoparçacıkların sıvı içerisindeki ve yüzeydeki düzenlenmesini çalıştık. Peptid yükünün altın nanoparçacık düzenlenmesinde çok büyük bir etkisi olduğunu bulduk. Bu çalışmadan elde edilen bilgi nanoparçacık düzenlenmesinde daha basit ve yeni tekniklerin bulunmasına yardım edebileceğini düşünüyoruz.

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LIST OF SYMBOLS / ABBREVIATIONS

AFM	Atomic force microscope
AgNPs	Silver nanoparticles
AuNPs	Gold nanoparticles
CNTs	Carbon nanotubes
DLS	Dynamic Light Scattering
DNA	Deoxyrubonucleic acid
FTIR	Fourier transform infrared
MEMS	Micro-Electro-Mechanical Systems
MNPs	Magnetic nanoparticles
NPs	Nanoparticles
QDs	Quantum dots
SAXS	Small angle X-ray scattering
SERS	Surface enhanced Raman scattering
TEM	Transmission electron microscope
UV	Ultra violet

1. INTRODUCTION

Nano as a word comes from the ancient Greek word *nanos*, which means dwarf. Today, nano (nm) refers to a length unit, which is the one billionth of a meter. Most recently, it is mostly associated with the field of nanotechnology. Figure 1 shows different sized structures from nano to micrometer.

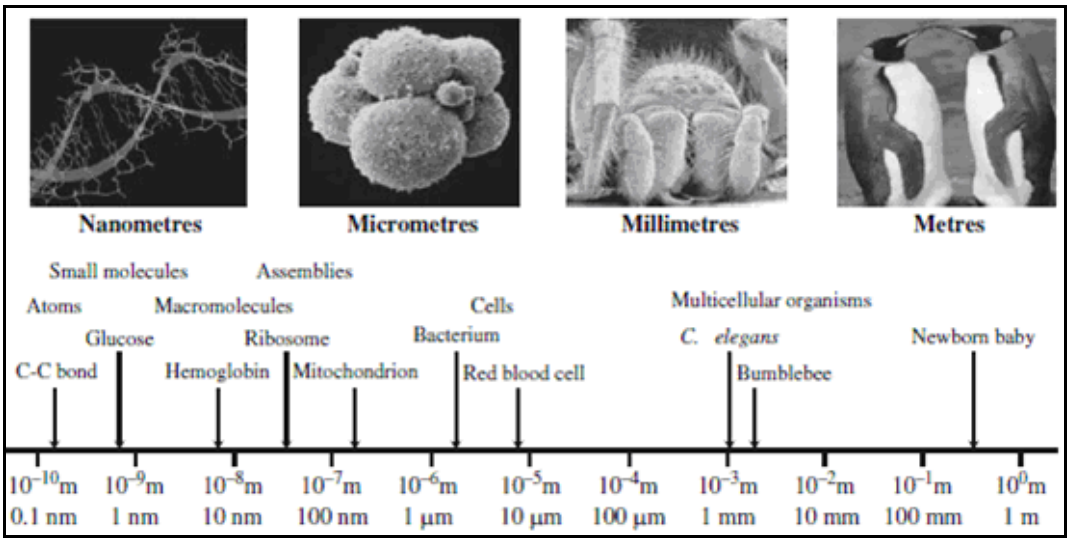

Figure 1.1. The length of structures from nano to macro [1]

The realm of nanoscience is not new. Human beings have been using it for centuries. They have started to use the gold colloids nearly 4th and 5th century B.C. in Egypt and China [2]. The gold colloids were used to stain the glasses and ceramics. The most famous example of these is the Lycurgus Cup, which was manufactured in near 5th century B.C. It is ruby red in transmitted light and green in reflected light. In medieval ages, the gold colloids were also used for curing of diseases [3]. In 1857, Faraday reported that after the reduction of the aqueous solution of chloroaurate ($AuCl_4^-$) by phosphorus in CS_2, a deep red colored colloidal gold suspension was formed [4]. He also worked on the optical properties of the colloidal gold [4]. The newness of today's nanotechnology and nanoscience concept is the heavy focus of nanotechnology and nanoscience on the developing applied technologies. It can be said that after the famous talk of Richard Feynman in 1959, "There is Plenty of Room at the Bottom", nanotechnology as a new and

contemporary concept started to emerge [5]. In his talk, Richard Feynman talked about constructing structures by manipulating the atoms and molecules and notably about the size change, important forces like gravity, friction forces etc. becomes less important and forces like surface tension and Van der Waals becomes more significant. After 15 years, in 1974, a Japanese scientist Norio Taniguchi used the term nanotechnology. This was the first time that the term "nanotechnology" appeared in press. In his paper, he defined the term as follows: "Nano-technology mainly consists of the processing of, separation, consolidation, and deformation of materials by one atom or by one molecule" [6]. In 1980s, Eric Drexler deepened the definition of the nanotechnology and he promoted the technological significance and importance of the nanoscale phenomena and devices by his works. His book "Engines of Creation: The Coming Era of Nanotechnology" is considered as the first book which has been written about the topic of nanotechnology. The research in nanotechnology and nanoscience accelerated after the invention of the Scanning Tunneling Microscope (STM) in early 1980s and later with the invention of AFM. In recent years, a new branch of nanotechnology called nanobiotechnology has been also emerged with the developments in the fields of nanotechnology and biology. In this field, scientists attempt to use nanotechnology in biological applications or try to combine the structures produced by nanotechnology with biological structures.

In this study, we investigated the assembly and behavior of the peptide modified gold nanoparticles in suspension and on surfaces. Little is known about on how peptides control the assembly of nanoparticles. There are very few data about how peptides influence of nanoparticle assembly. Also, little is known about the novel properties of the peptide-nanoparticle hybrid systems. Therefore with this study, we aimed to develop better understanding on the properties of peptide-gold nanoparticle hybrid systems.

2. THEORETICAL BACKGROUND

In this part, general information about nanotechnology, nanoparticles (NPs) and ways to construct the nanostructures are provided.

2.1 NANOTECHNOLOGY

Nanotechnology is commonly defined as the understanding and control of matter at sizes between approximately 1 and 100 nanometers. However, this definition is somewhat inadequate. Nanotechnology is not only about the size but also that the structures produced have novel features. From the website of the National Nanotechnology Initiatives (NNI), nanotechnology is defined as "the understanding and control of matter at dimensions between approximately 1 and 100 nanometers, where unique phenomena enable novel applications". In nanometer size scale, the matter behaves differently from its bulk state. Normally, in their bulk state, materials are under the influence of Newton mechanics. When the size of materials decreases to nanometers, the materials are under the influence of Quantum mechanics instead of Newtonian mechanics and this event is called the Quantum size effect. The quantum size effect is the unusual properties of extremely small crystals arising from the confinement of the electrons to a restricted region [7].

2.2. NANOPARTICLES

Nanotechnology has had a massive effect on a variety of scientific disciplines ranging from catalysis to biotechnology. Nanoparticles such as gold (AuNPs), silver nanoparticles (AgNPs), carbon nanotubes (CNTs) and quantum dotes (QDs) are at the top of rapidly increasing list of materials being investigated in nanostructured form. Recently, these NPs have been widely used due to their magnetic, electronic and optical properties which are different in their nano sized form. These properties of nanoparticles change with the size shape and the medium which synthesized the substance. Since they have these extraordinary properties, scientists try to use them in inventing novel chemical and biological sensors, optical and eletro-optical devices, high capacity data storage devices and substrate for SERS [8]. To obtain these NPs, different synthesis techniques are used

such as laser ablation, reduction by ultrasound, Gamma ray, UV-ray and reduction with the organic and inorganic agents [8]. Below brief information about some nanostructures will be provided.

2.2.1. Carbon Nanotubes

Carbon nanotubes (CNTs) which can be seen in Figure 2.1 are graphitic sheets with hexagonal lattices which are wrapped up into cylinderic form. They have attracted the attention of the physicists, chemists and material scientists due to their intriguing electronic, magnetic, optical and mechanical properties. They have unusual shapes and sizes. They can be single walled or multi walled. They can have a very high length to diameter ratio (can be up to 132.000.000:1) [9]. Scientists try to use CNTs in different areas. Due to their electronic properties, most research for the use of CNTs focuses on electronics and nanodevice construction [10]. Their use as hydrogen storage, receptor phase in chemo and biosensing and delivery agents in medicine has also been investigated [11-13].

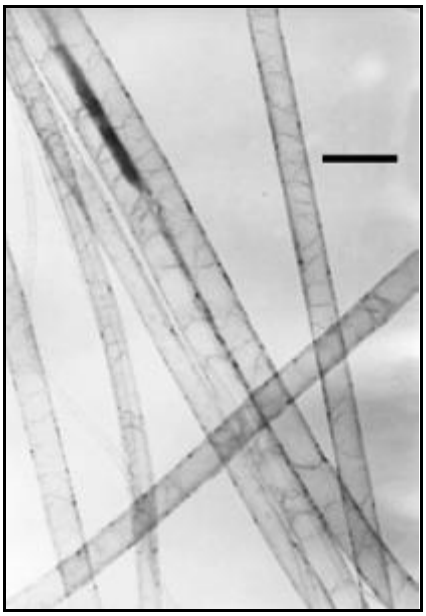

Figure 2.1. TEM image of CNTs [14]

2.2.2. Quantum Dots

Quantum Dots (QDs) are semiconductor nanoparticles that have only a small number of free electrons and these electrons are confined in a very small volume [15]. They were invented by the Russian scientist Alexei Ekimov [16]. Their size changes with the synthesis technique and their size ranges in between 2 to 10 nm. They are widely studied in nanotechnology due to their extraordinary photochemical and photophysical properties. They emit light with different wavelength depending on their size as can be seen in Figure 2.2. The fluorescent emission of QDs can be adjusted by the changing the size, shape and composition [17]. Since they are structurally and fluorescently more stable than the common organic dyes and fluorescent proteins and their capability to emit multicolor fluorescence, they are starting to be used in in vivo studies in place of these dyes and proteins [18, 19]. QDs can be used for cellular imaging. Dvorak and his group used QDs to visualize the erythrocytes [19]. Dahan and his group used QDs to track individual glycine receptors and they analyzed their movement in neural membranes for periods ranging from milliseconds to minutes [20]. Also, scientists have studied to use of QDs in transistors, solar cells, diode lasers and LEDs [21-23].

Figure 2.2. Different sized quantum dots [24]

2.2.3. Magnetic Nanoparticles

Magnetic nanoparticles (MNPs) are another class of nanoparticles that allow the manipulation by using magnetic fields. They can be synthesized from iron oxides, metals such as iron, cobalt or nickel and from alloys that contain iron cobalt etc [25]. The sizes of the particles range from 1 to 100 nm. There are some problems in synthesis and the most important one is the synthesis of water soluble MNP due to their stability in biological systems [25]. So, the MNPs are coated with different molecules such as oleic acid, hexadecanediol [26]. MNPs can be used as magnetic fluids which are also known as ferro fluids and have a wide application area in industry. These ferro fluids are used in audio speakers and high speed computer drives to remove impurities. Scientists try to use MNPs in catalysis, magnetic resonance imaging, data storage and bioremediation [27-30].

Figure 2.3. TEM image of assembled magnetic nanoparticles [31]

2.2.4. Silver Nanoparticles

Silver nanoparticles (AgNPs) are the one of the hottest topic in nanoparticle researches due to their plasmonic and antibacterial properties. These properties of the AgNPs change with size and shape. There are various ways to synthesize AgNPs and the easiest and most common techniques are citrate and $NaBH_4$ reduction methods [32, 33]. In Figure 2.4., AgNPs synthesized by using citrate reductiom method is seen. Uniform particle synthesis and shape control are the most challenging problems in AgNP synthesis. Recently, Mirkin and his co-workers found a way to synthesize uniform silver nanoprisms

[34]. Different shaped silver nanoparticles such as triangular silver nanoplates, nanocubes can also be synthesized by using different chemicals [35, 36].

Silver nanoparticles can be used in different applications. AgNPs are commonly used as Surface Enhanced Raman Scattering (SERS) substrates due to their plasmonic properties [37, 38]. They have been used for the detection of microorganisms and certain chemicals [39]. AgNPs have antimicrobial effects so there are many ongoing studies about using them in the textile and dye industries [40, 41]. It was also found that AgNPs can have catalytical activity [42].

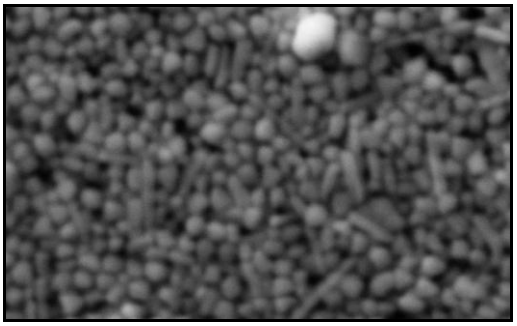

Figure 2.4. SEM image of the silver nanoparticles

2.2.5. Gold Nanoparticles

AuNP seen in Figure 2.5. are another commonly studied nanoparticle type. They demonstrate fascinating aspects such as size related electronic, optical (quantum size effect) properties, the behavior of the individual particles and their applications to catalysis and biology [2]. AuNPs are the most stable type among metal nanoparticles [2]. They are promising materials in bottom up approach in nanotechnology. There are many ongoing studies about AuNPs. Scientists try to apply AuNPs in different areas.

It is predicted that the use of soluble gold started in Egypt and China in the 4^{th} and 5^{th} centuries B.C. They were used to stain glasses and ceramics. In the middle ages, colloidal gold started to be used for medical purposes [43]. In the 16^{th} century, Francisci Antonii wrote the first book about colloidal gold and its medical uses. Michael Faraday explained

the formation of the colloidal gold in 19th century. Now in the 21st century, a lot is known about colloidal gold.

There are several approaches reported in the literature for synthesis of AuNPs with different shape and size [44-48] Scientists have been trying to synthesize AuNPs in different ways and they have been trying to synthesize different sized and shaped AuNPs. In the past several decades, many developments have been done in production of AuNPs. Among these, the Turkevich method can be seen to be very important. With this technique stable and uniform AuNPs can be produced in aqueous environments [44,45]. In the Turkevich method, diluted aqueous solution of $HAuCl_4$ is reduced by using trisodium citrate to form the AuNPs. The size of the AuNPs depends on the ratio between the amount of reducing agent and the amount of $HAuCl_4$. The AuNPs can also be synthesized in different shapes like rod shaped, triangular shaped and cube shaped which have different optical and magnetic properties by using different techniques and chemicals [46-48].

In recent years, scientists have attemped to use AuNPs in different areas such as electronics, sensing, biomedicine and nanoconstruction [49-52]. The unique optical properties of AuNPs and easy shape manipulation and surface modification make them promising materials. The formation of surface plasmons upon their interaction with light make AuNPs attractive building blocks for nanoscale electronic and photonic devices as well as signal transducers and signal amplifiers in a variety of biosensing platforms that exploit colorimetric, surface-enhanced Raman scattering (SERS), fluorescent, and electrochemical assays [53, 54].

One of the promising use of AuNPs is the AuNP-based colorimetric biosensor, which takes advantage of the color change that arises from the interparticle plasmon coupling during AuNP aggregation (red to blue) or redispersion of an AuNP aggregate (blue to red) [55]. The first DNA sensor that have worked in this principle was made by Mirkin group and after that study, the technique has been increasingly applied to detect a large variety of biological targets such as DNA, peptides, saccharides, small molecules, metal ions and cells [56-58]. This technique is an alternative method to the current fluorescent based technique and it can be used in clinical diagnostics, drug discovery and environmental contaminant analysis.

AuNPs are also used in medicine. They can be used in imaging of biological systems [59]. Also they can be used as therapeutic agents [60]. Photothermal therapy can be given as an example. Gold nanorods are used in this therapy [60]. The gold nanorods are given to the cancer area and they are heated by IR radiation. Then the light is converted to heat by the nanorods kills the cancer cells.

Another promising use of AuNPs is the construction of nanostructures by using bottom up approach. Scientists use AuNPs to construct nanostructures by using different methods [61, 62]. One method is to modify the nanoparticles with different ligands such as DNA, peptides etc. to benefit the affinity force between the molecules so that the modified AuNPs can form the desired and predetermined shapes. Also, in manufacturing bigger structures from AuNPs, different shaped NPs were also synthesized and used [63].

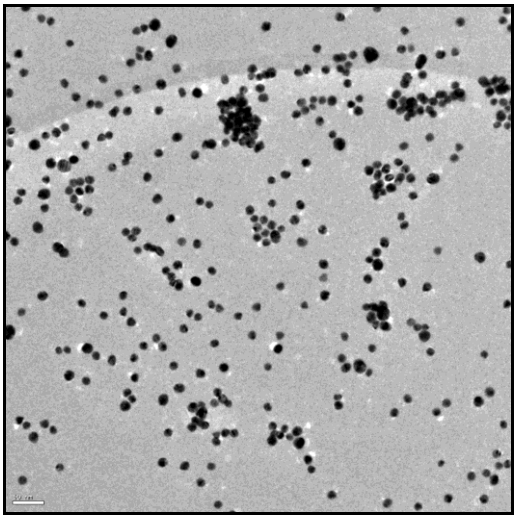

Figure 2.5. TEM image of gold nanoparticles

2.3. CONSTRUCTING NANOSTRUCTURES

Although there is enormous progress in nanoscience and nanotechnology, there are still major problems with the synthesis of uniform size and shape of particles and constructing the higher order structures using the nanoparticles as building blocks. For the first problem, scientists have found many ways to synthesizing nanoparticles and the problem has been overcome to a certain degree [64-66]. However, second problem is the

real challenge for scientists in variety of scientific fields. There are 2 main approaches to manufacture the nanoconstructions namely top-down and bottom-up which are schematically shown in Figure 2.5.

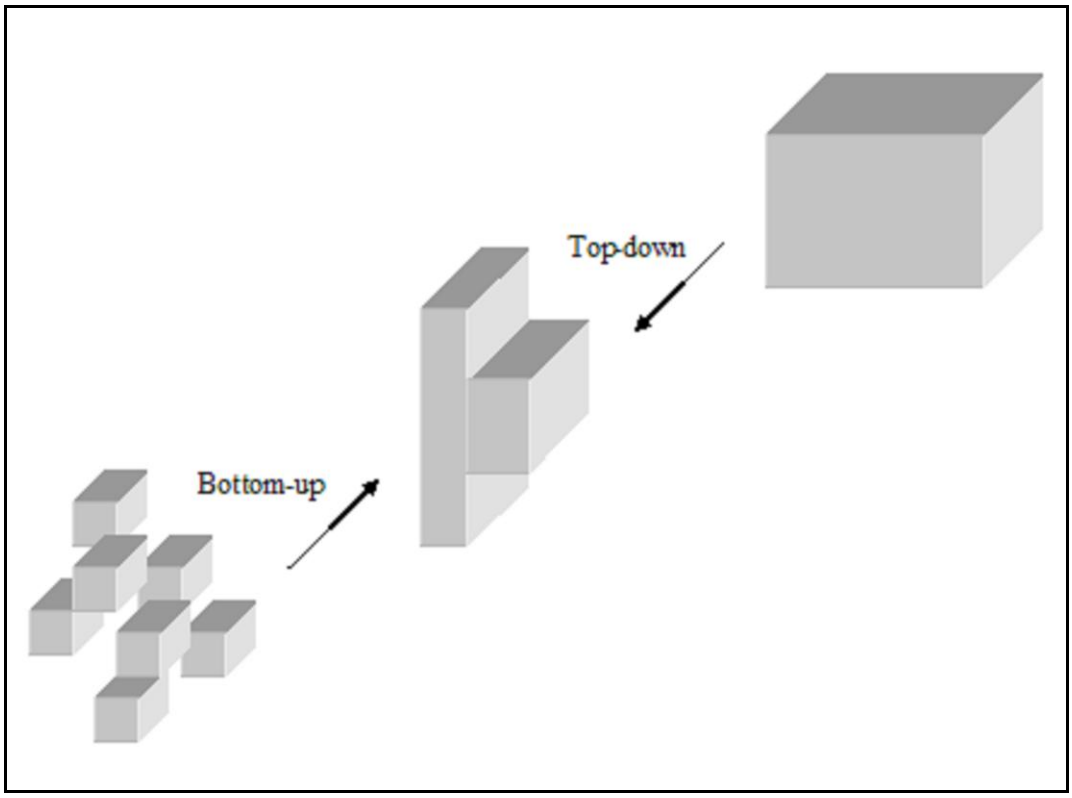

Figure 2.6. Schematic representation of Top-down and Bottom-up approaches

2.3.1. Top-down Approach

In top-down approach, small particles are manufactured from the bulk materials by using external forces. Tools are used to cut, mill and shape the materials into desired size, shape and order.

For construction of micrometer size structures, lithography is commonly used in science and technology. Litography refers to a group of technique that uses a template to generate the desires pattern on surfaces. Here the process of transferring shapes on a mask to the surface of a silicon wafer is briefly outlined for clarity of lithographic process. This process is also used for the fabrication of microchips and MEMS [67, 68]. The procedure

consists of several steps in sequences, which are namely wafer cleaning, barrier layer formation, photoresist application, soft baking, mask alignment and exposure. In the first step, the surface of the wafer is cleaned from any organic and inorganic wafer. After the cleaning step, as a barrier layer silicon dioxide (SiO_2) is deposited on the surface of the wafer. After this, by using spin coater, a photoresist material is applied to the surface of the wafer. Then, the part called soft baking takes place. In this part, the soluble part of the SiO_2 washed away. Since photoresist becomes photosensitive, the soft baking is very important. After that the mask has to be aligned before the high intensity UV exposure.

There are also other lithographic techniques such e-beam lithography uses electron source instead of light source [69] and dip-pen lithography using a AFM tip. No mask is needed in this type of lithography but it is slower and expensive than the concentional lithographic techniques. X-ray lithography uses x-ray to form the surface [70]. It is again very expensive. Ion beam lithography uses ionized ions [71].

Although there are many ongoing researches and applications about lithography, many drawbacks are found in this technique. Smallness is one of the biggest problems in lithography. Too small patterns could not be produced due to the resolution of the light source in photolithography. It is not easy precisely to locate the nanoparticles to the desired locations. Imperfection at the pattern edges is another drawback. Also the instruments used in the lithography are very expensive and the technique is very time consuming.

2.3.2. Bottom up Approach

The second approach is called bottom-up and refers to the constructing the higher structures and patterns starting using nanoparticles as building blocks. In bottom up approaches, chemical properties of the molecules are used to bring the nanoparticles into the desired locations in the structure. The well-established molecular self assembly is utilized for the bottom up approach and this is achieved by modifiying the surface properties of nanoparticles with variety of molecular structures. Self assembly is a process in which the achievement of the spontaneous organization of the discrete components to ordered and/or functional superstructures without any outside force intervention [72]. There are mainly two types of self assembly namely equilibrium self assembly and

dynamic self assembly considering the thermodynamic description of the resulting structural assemblies.

Self assembly is a very easy way to construct the desired nanostructures. There is no need for complex and expensive instruments. Also, with respect to top to bottom approaches, the process is very fast. Thus, bottom up approach is an elegant alternative method to “top-down” approach.

2.3.3. Assembly of Nanoparticles

To create functional devices, which can be used in nanoelectronics, spintronics and photovoltaics applications, precise assembly and patterning of nanostructures is essential. Self assembly is an opportunity for constructing such functional devices since it simplifies and reduces the manufacturing processes [73]. The assembly of NPs into the desired organizations and patterns is critically important for construction of higher structures using nanoparticles as building blocks. Below, different methods nanoparticle assembly and principles of these processes are summarized.

2.3.4. Assembly in Liquid-Solid Interface

In these types of assemblies, assembly of the nanoparticles is performed on a surface after the liquid part of the nanoparticle suspension is evaporated.

2.3.4.1. Drying mediated Assembly

It is the simplest method for assembling nanoparticles on surfaces, which is also called evaporation mediated assembly demonstrated in Figure 2.7. Nanoparticles are forced to assemble as solvent evaporates. The weak attraction forces among the nanoparticles become significant with the slow evaporation of the aqueous phase which forces the nanoparticles to organize. This type of assembly was first elucidated by the Denkov and coworkers. Pileni et al. explained the structure formation mechanism of Ag_2S nanoparticles [74]. In this type of assembly, it is initially thought that the nanoparticles are well dispersed in the suspension. As soon as the evaporation starts, the concentration of the nanoparticles in the suspension begins to increase. Since the evaporation rate is faster than

the nanoparticle diffusion, the particles are assembled on the surfaces. This method depends on the electrostatic interaction between nanoparticles, capillary forces, morphology (size and shape) of the nanoparticles [75]. There is also a phenomenon called "coffee ring" which is the concentric rings of nanoparticles formed with the evaporation of nanoparticle suspension [76]. It is newly reported that with outside force, these rings can be manipulated [77]. Thus, it can be concluded that drying mediated assembly method provides a rapid, cheap and simple way to assemble various types of nanoparticles.

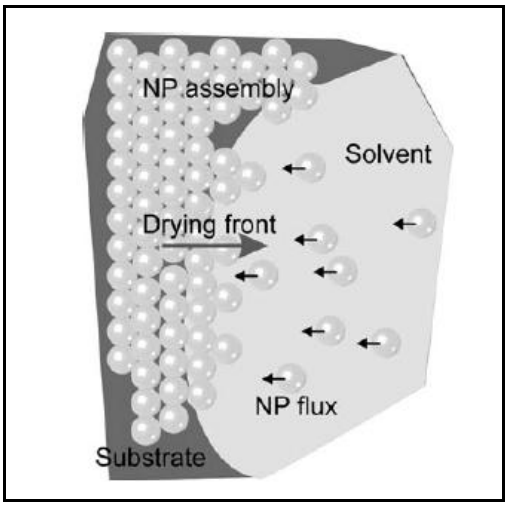

Figure 2.7. Drying mediated self assembly on surfaces [78]

2.3.4.2. Template Assisted Assembly

Assembly of the nanoparticles on the patterned surfaces is a widely used method. The patterned surfaces obtained by the lithographic techniques such as soft-lithography, e-beam lithography and light lithography are used for directing the nanoparticle assembly or increasing the assembly rate [79-81].

2.3.4.3. Programmed Self Assembly

Among the self assembly techniques, programmed self assembly is seen as the most promising one. Nanoparticles are modified with certain molecules that have strong affinity to each other. DNA and peptides are the biological molecules that can be used for programmed assembly [82, 83]. There is a huge research effort ongoing about programmed assembly. Mirkin et al and Alisivatos et al have showed that complimentary DNA oligodeoxynucleotides can be used as assembling agents for the nanoparticles [84, 85].

There are also a number of reports in assembly of DNA modified nanoparticles [86-89]. Scientists also try to use other biomolecules for the programmed assembly such as RNA, lipid, carbohydrate and some small molecules for constructing the nanostructures [90-92].

Recently, scientists used peptides as assembling agents for the nanoparticles. However, since there are many parameters that affect the peptide structure and conformation, working with peptides and peptide modified nanoparticles is very difficult. Levy et al. tried to design oligopeptides that can stabilize the gold nanoparticles in suspension without aggregation and also used specific molecules for molecular recognition [93]. They systematically tried 58 different peptides and found that the most stable peptide was the peptapeptide CALNN. Porta et al. synthesized some specific peptides and investigated the behavior of the peptide modified AuNPs on hydrophilic surfaces [94]. They used cysteine and lysine containing peptides and they used them as capping agents in the preparation of monolayer protected (MPC) AuNPs. Bishnoi and her group used gold binding peptides and investigated the assembly of peptide modified AuNPs in suspensions and on hydrophilic surfaces [95]. A study done by the Rosi and his group showed that by using peptides the nanoparticles can be assembled as nanoribbon as showed in Figure 2.8 [96]. They used peptides which has the amino acid sequence of AYSSGAPPMPPF. In an other study, the behavior of the peptide modified AuNPs were studied with different pHs and different concentrations of zinc ions [97]. Behavior of Gluthathione modified AuNPs were also investigated with different pHs [98]. Mandal and his group studied the effect of pH on the assembly of the peptide modified AuNPs [99].

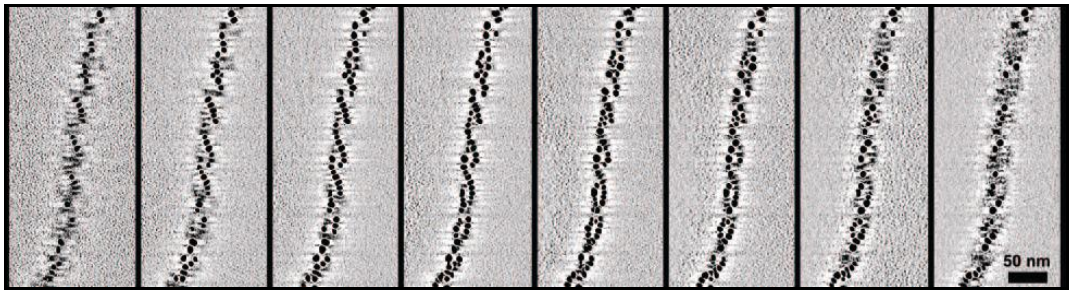

Figure 2.8. TEM image of the AuNP double helices [98]

In this study, with the knowledge obtained from the literature, anionic and cationic peptides which are different in size and charge were designed. Peptides can bind AuNP surface with their thiol groups which are found at the N-terminal cystein of the peptides. After the modification, behavior of the peptide modified AuNPs were investigated in suspension and on hydrophilic surface.

3. MATERIALS

3.1. REAGENTS

$HAuCl_4.3H_2O$ was purchased from Fluka (Germany) and sodium citrate ($NaH_2C_6H_5O_7$) was purchased from Merck (USA). Peptides which can be seen in Table 3.1 and Figure 3.1 and Figure 3.2 were purchased from CPC Scientific (USA). 10K MWCO Slide-A-Lyzer Dialysis Cassettes were purchased from Thermo Fisher Scientific Inc. (USA).

3.2. PEPTIDES

All the peptides were purchased for modifying the surface of the AuNPs. The peptides were designed as anionic or cationic when they are dissolved in dH_2O. All the peptides were purchased as lyophilized and 10 mg. Also all the peptides have a cystein in their N-terminal for gold binding (Au-S- bond). The 5 of 13 peptides are designed as anionic and 8 are cationic at pH 6.

Table 3.1. The properties of peptides used in the studies

Code	Peptide	Charge	Number of Amino acids
P1	C-S-E	-	3
P5	C-S-E-D-S-D	-	6
P16	C-S-E-D-S-D-E-S-D-S-E-S-D-S-E	-	15
P18	C-C-S-E-D-S-D-E-S-D-S-E-S-D-S-E-S-D-S	-	19
P20	C-C-S-E-D-S-D-E-S-D-S-E-S-D-S-E- S-D-S-E-S	-	21
P2	C-K-R	+	3
P6	C-K-R-H-S-K	+	6
P10	C-K-R-H-S-K-R-H-R	+	9
P14	C-K-R-H-S-K-R-H-R-S-K-R	+	12
P17	C-K-R-H-S-K-R-H-R-S-K-R-H-S-K	+	15
P19	C-K-R-H-S-K-R-H-R-S-K-R-H-S-K-R-H-S	+	18
P21	C-K-R-H-S-K-R-H-R-S-K-R-H-S-K-R-H-S-K-R-H	+	21
P23	C-K-R-H-S-K-R-H-R-S-K-R-H-S-K-R-H-S-K-R-H-S-K-R	+	24

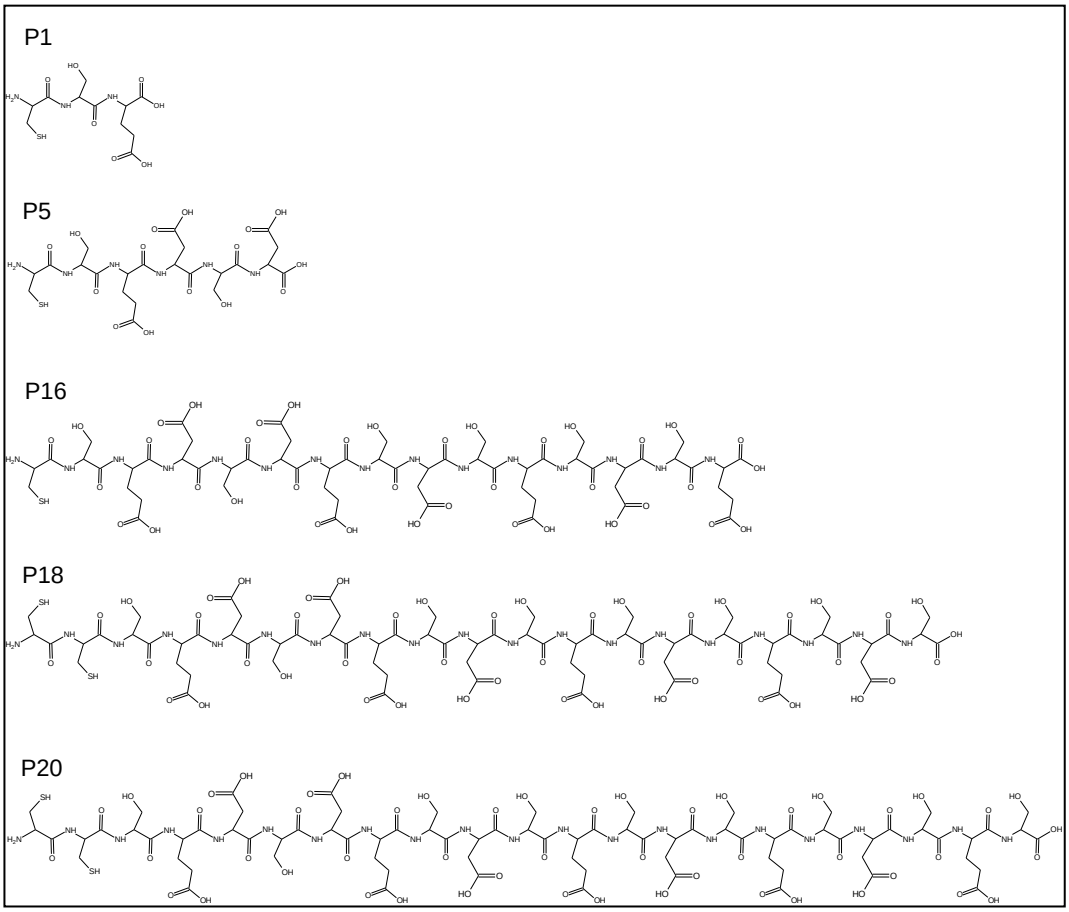

Figure 3.1. Schematical representation of anionic peptides used in the study

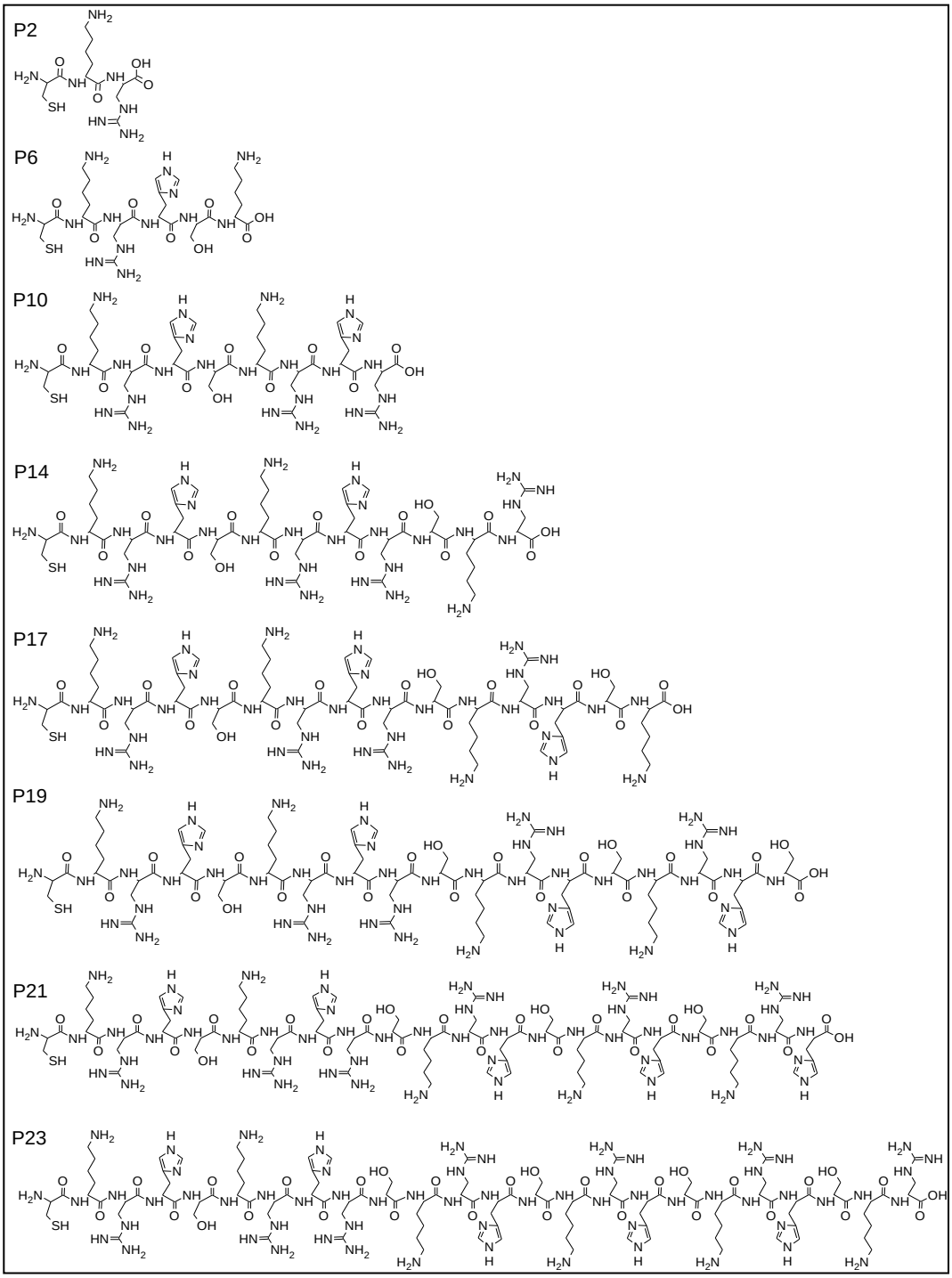

Figure 3.1. Schematical representation of cationic peptides used in the study

4. METHOD

4.1. PREPARATION OF PEPTIDES

The purchased lyophilized peptides were dissolved in distilled water. Their stock solutions at 10^{-3} and 2 x 10^{-4} M were stored at -20^0C. Capital P is used as an abbreviation and the peptides are coded as P1, P2, P5, P6, P10, P14, P16, P17, P18, P19, P20, P21 and P23.

4.2. SYNTHESIS and CHARACTERIZATION OF AuNPs

AuNPs were prepared by reduction of $HAuCl_4 \cdot 3H_2O$ with sodium citrate. This procedure generates an average size of 13 nm gold nanoparticles. Briefly, 500 ml of 1 mM $HAuCl_4 \cdot 3H_2O$ solution was prepared. The $HAuCl_4 \cdot 3H_2O$ solution was heated until to boiled, and then 50 ml of 38,8 mM citrate stock solution was added into it. The final solution was kept boiling for 15 min. After cooling to room temperature, the suspension was filtered using a 0.45 μm filter. For characterization, UV-Visible Spectroscopy, Dynamic Light Scattering (DLS), Atomic Force Microscope (AFM) and Transmission Electron Microscope (TEM) are used where necessary.

4.3. MODIFICATION OF THE NANOPARTICLES WITH PEPTIDES

The AuNPs were modified with both anionic or cationic peptides. In the literature, different numbers are reported for the maximum number of peptides in which a 13 nm AuNP can be loaded [93, 94]. Since we have peptides with different sizes and different charges, we attempted to modify the AuNPs with different concentration of the peptides. Finally, we decided to add ~300 peptides per nanoparticles. We chose this number due to the fact that the positively charged peptide modified AuNPs were not stable when we increased this number further. After the addition of the peptides, nanoparticle suspension was incubated for 24 hours.

4.4. DIALYSIS of MODIFIED AuNP SUSPENSION

After the incubation period a dialysis procedure was performed to the gold-peptide suspension to remove unbound peptides and unbound ions such as citrate and Cl^-. The dialysis membrane allowed only unbound peptides and ions to pass through since the peptide bound AuNPs are larger than 10 kDa and cannot pass the membrane. The use of dialysis cassette as follows; 10 ml of peptide modified AuNP suspension injected into the dialysis cassette with an injector and the cassette placed into distilled water in a 2-litre beaker. The water was changed in every 2 hours and this procedure was repeated 3 times. After the last water change, the beaker was put into the refrigerator and kept there overnight at $+4^{O}C$ to increase the efficiency of the dialysis by slowing down the dialysis process.

4.5. ANALYSIS of PEPTIDE MODIFIED AuNPs

After modification and dialysis, the pH of the peptide modified AuNPs were measured and after that, the assembly of the peptide modified AuNPs analyzed when they were in suspension and on hydrophilic surface.

4.5.1. Zetasizer Analysis

Size distribution and surface charge of the nanoparticles were measured by using a Malvern Zetasizer Nano ZS (Malvern, UK) at 25°C. It contains a 4 mW He-Ne laser which has a wavelength of 633 nm and it has an avalanche photodiode detector. The scattered light was detected at an angle of 173°.

4.5.2. FTIR Analysis

Thermo Scientific FTIR instrument was used for FTIR analysis. ATR mode was used in the study. The data were taken as absorbance. The dehydrated powder of the peptide modified AuNPs were used.

4.5.3. Raman Instrumentation

To perform all SERS experiments a Renishaw inVia reflex Raman microscopy system was used. The system was automatically calibrated against a silicon wafer peak at 520 cm^{-1}. A diode laser at 830 nm and a ×50 objective (numerical aperture is 0.75) with a laser power of 30 mW were used in all experiments.

4.5.4. AFM Analysis

A Park SYSTEMS XE 100 Atomic Force Microscope (Park Systems Corp., Korea) was used for AFM analyses. A non-contact silicium tip was used in the analyses. The scan rate was 0.5 Hz.

4.5.5. TEM Analysis

TEM measurements were performed with JEOL-2100 HRTEM operating at 120 kV (LaB_6 filament) and equipped with an Oxford Instruments 6498 EDS system.

5. RESULTS & DISCUSSION

The unmodified and peptide modified AuNPs were characterized and their behavior in suspension were investigated. Then, their behavior on the hydrophilic surfaces were investigated as the solvent dries from the droplet.

In the study, the 13 nm AuNPs were selected since they can relatively easily be synthesized in uniform size. AuNPs, especially 13 nm AuNPs, are one of the most stable NPs synthesized in suspension. Furthermore, the AuNPs were selected since their behavior in suspension can be easily traced by colorimetric detection and the mode of their interaction with peptides using SERS. Finally, AuNPs are easily modified through well established and characterized Au-S- bonds [93]. It is also known that Au surfaces have an affinity for NH_2 moieties [100].

5.1. Characterization and Modification of the Gold Nanoparticles

5.1.1. Characterization of Unmodified Gold Nanoparticles

Figure 5.1 provides the UV/Visible spectrum, Figure 5.2 provides the DLS plot and Figure 5.3 provides TEM image of the unmodified AuNPs. The maximum absorption of the 13 nm AuNP suspension is measured at 520 nm. For further confirmation of the size of the AuNP suspension and to determine the size distribution of the colloidal suspension, Zetasizer was used. As seen in Figure 5.2, the size of the colloidal gold nanoparticles is nearly 13 nm. The TEM image in Figure 5.3. shows that the size distribution is consistent with previous data.

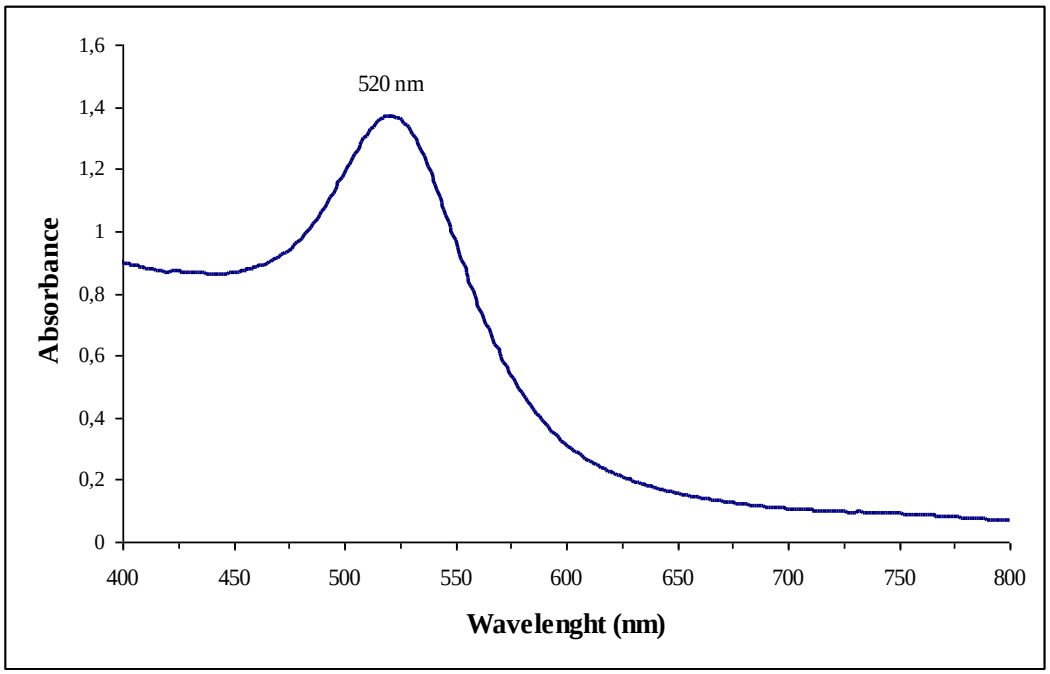

Figure 5.1. UV/Visible Spectrum of the unmodified 13 nm AuNPs

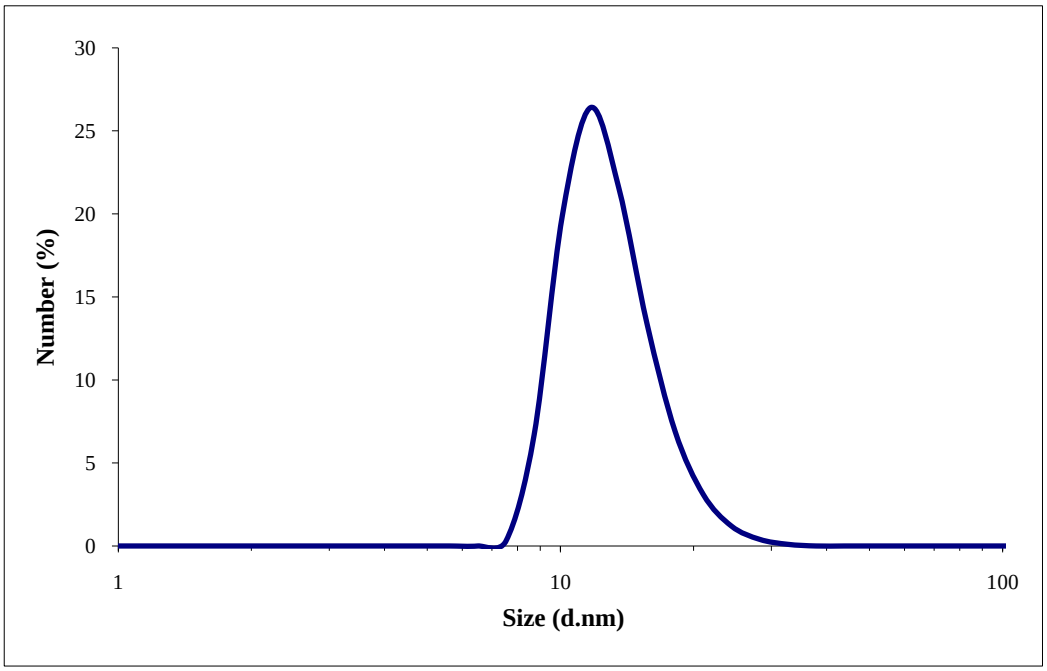

Figure 5.2. Number size distribution of the colloidal unmodified gold suspension

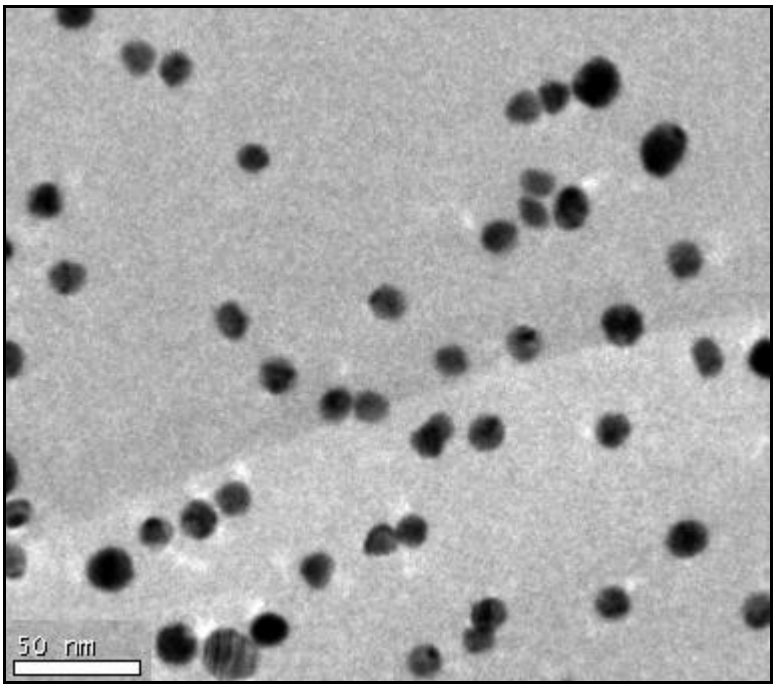

Figure 5.3. TEM image of AuNPs

5.1.2. Peptide Modification of the AuNPs

Table 3.1 and Figure 3.1 show the sequences and the chemical structure of peptides used in this study. The peptides are grouped into two categories; negatively charged and positively charged. Each peptide carries a cystein moiety on one end to interact with Au surface. The size and charge on the peptide systematically changes to be able to observe the possible effect on the assembly in suspension and at interfaces. The peptides may also interact with AuNPs through their N-terminus, C-terminus or other functional groups present in their structures. Figure 5.4 shows the representation of possible interaction between AuNPs and negative (A) and positive (B) peptides. It is known that amino ($–NH_2$) and thiol (-SH) groups can interact with the AuNP surface [93, 100]. Since all the peptides used in this study possess at least a cystein in their N terminal, the peptides were thought to bind the AuNPs through Au-S bond. The charge of the peptides is gradually increased on both anionic and cationic sequences. For the anionic peptides the ionizable –COOH groups are responsible for the negative charge and P1, P5, P16, P18 and P20 possesses 2, 4, 9, 10 and 11 ionizable –COOH groups including C-terminus, respectively. For the cationic peptides the ionizable $–NH_2$ groups are responsible for the positive charge and P2, P6,

P10, P14, P17, P19, P21 and P23 possesses 3, 5, 8, 10, 12, 14, 17 and 19 ionizable $–NH_2$ groups including N-terminus, respectively.

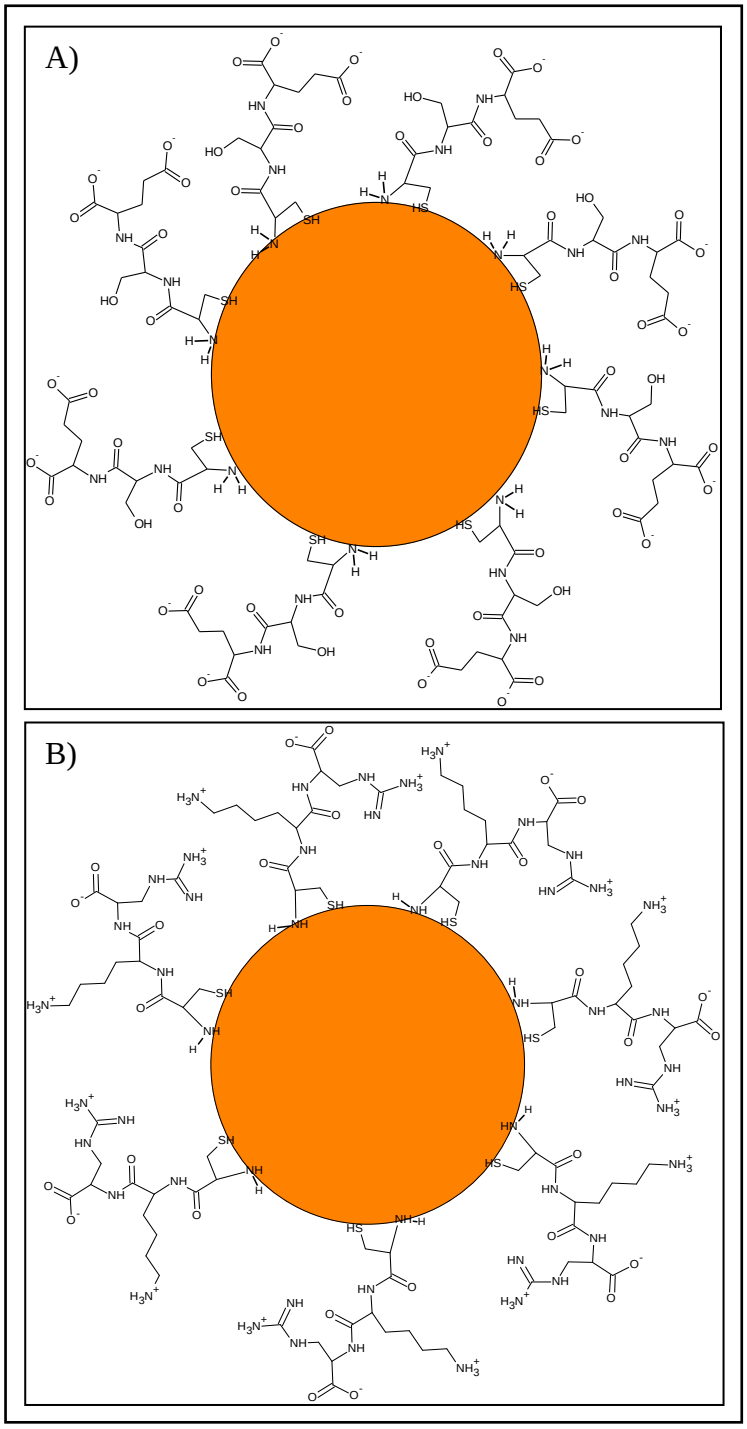

Figure 5.4. Schematical representation of the peptide bound AuNPs A) P1 bound B) P2 bound

It is estimated that the number of the AuNPs in 1 mL after the citrate reduction method is 10^{12} [101]. In order to load as many peptides as possible onto the surface of the AuNPs, excess amounts of peptide molecules were added to the AuNP suspension. The number of peptide molecules theoretically estimated by dividing the surface area of 13 nm AuNP to the area occupied by a thiol bond (21,4 $Å^2$) [102]. At the beginning of the study, peptides were added in such an amount that on one NP, theoretically all the surface of the NP was covered. No problem was observed in anionic peptide modified AuNPs but in cationic peptide modification, excess addition of the cationic peptides caused immediate aggregation. The reason of this aggregation is thought that the negative citrate ions interacted with the cationic peptides and caused both surface charge instability and formed bridges which caused aggregation. The attraction force between the citrate and the amine groups could be an important factor to prevent complete migration of peptides during the dialysis. Therefore, the number of peptides added to the suspension decreased to 300 peptides on 1 NPs, which was resulted with stable colloidal suspensions.

5.1.2. Characterization of the peptide modified AuNPs

The pH of AgNP suspension is usually around 5.0. With the addition of the peptides, pH of the suspension containing modified AuNPs was measured as 6.0. The pH measurements were performed after dialysis. For the determination of the presence and confirmation of the peptide modification onto the AuNP surface, surface-enhanced Raman scattering (SERS) and FTIR spectroscopy were used.

In figure 5.5, FTIR data of the citrate capped and anonic and cationic peptide modified AuNPs can be seen. The data were obtained by using ATR mode of the FTIR instrument. Since peak coming from the water masks other peaks coming from different bonds, peptide modified AuNPs were dehydrated and the powder forms of them were used for the measurements. The data acquired from the citrate capped AuNPs is obviously different than the peptide modified AuNPs. In Figure 5.5.A, the peaks coming from the ~1630 cm^{-1} are assigned to the C=O from the carboxylic acid and in Figure 5.5.B, the peaks coming from the ~3270 cm^{-1} are assigned to the NH-CO mode of amide A [103, 104]. At these absorbances, scattering was seen due to the gold surface instead of the absorbance. It is thought that the lack of further peaks was due to the gold surface

interfering with the IR spectra of the bound molecules [105]. The IR spectra indicate that the peptides are chemically attached to the AuNP surfaces after the dialysis.

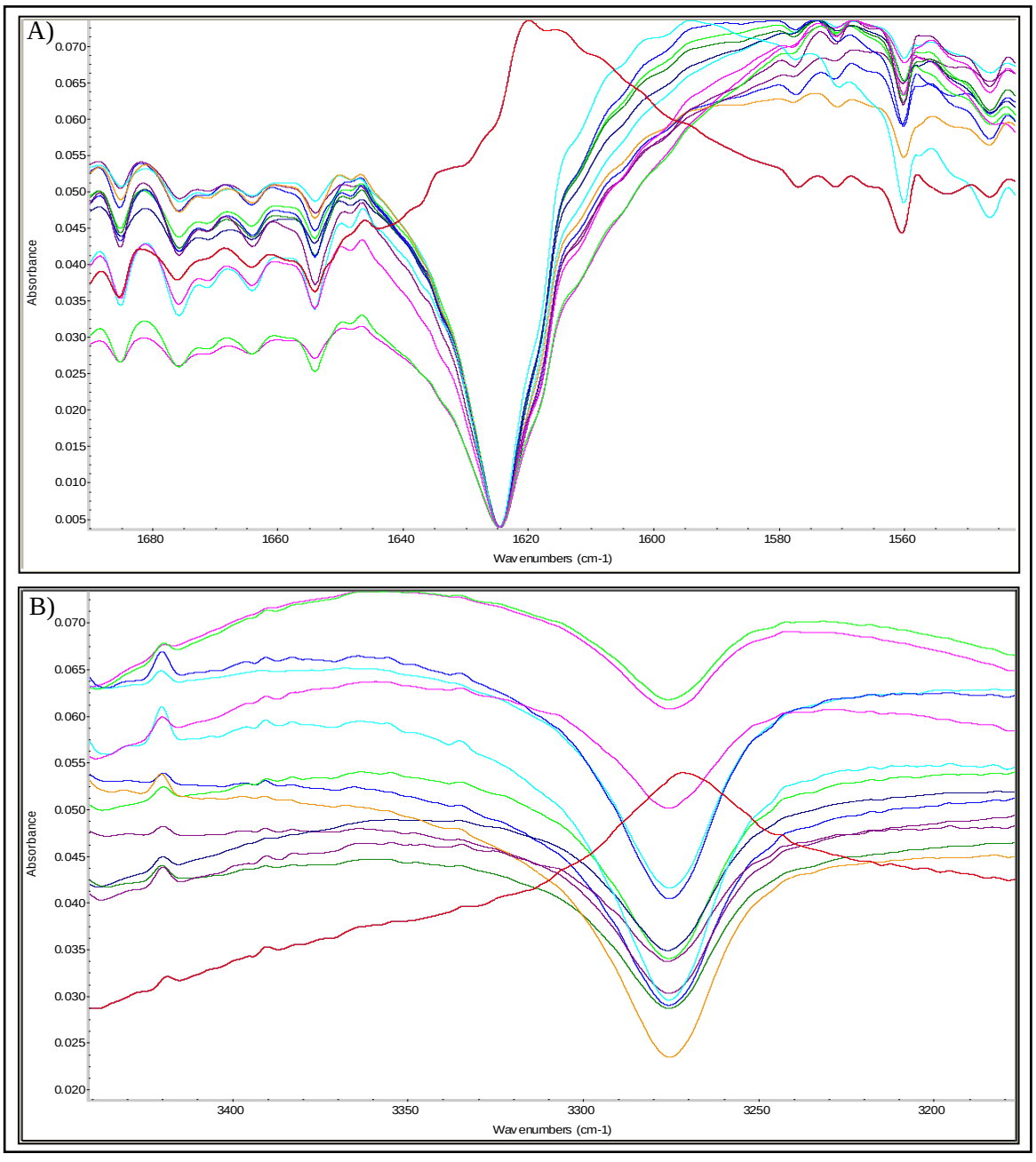

Figure 5.5. FTIR data acquired from the citrate capped and negaticveand positive peptide modified AuNPs A) interval between 1500-1700 cm^{-1} B) interval between 3150-3450 cm^{-1} (red line is the citrate capped AuPs)

Further, SERS was utilized to confirm the chemical attachment of peptides on AuNPs. Since Raman scattering of molecules are enhanced on noble metal surfaces such as

Au and Au, the spectra acquired from the peptide bound AuNPs can provide valuable information about the status of the peptides on AuNPs. Figure 5.6 shows the SERS spectra of peptide modified AuNPs. In a SERS experiment, the materials investigated should be 1-3 nm away from the metal nanoparticles [106]. We dropped the suspensions on calcium fluoride which gives nearly zero background spectra and investigate the spectra obtained from each suspension. In Figure 5.6, on both SERS spectra of both types of peptide-AuNP conjugates, the intensity of the peaks decreases as a general trend. This may indicate that the number of peptides in contact with AuNPs diminishes as their sizes increase. The peaks that appear in the interval of 1100-1200 cm^{-1} might originate from the N-H bonds, which are thought to be that the peptides are in a close proximity to the gold nanoparticles [105]. Although several peaks appeared on SERS spectra, on Au-S bond was not observed. According to the study done by the groups of Mantion and Taubert, they suggested that the metal-S bound cannot be unambiguously identified by using SERS [105]. In figure 5.6A, SERS data of the citrate capped and anionic peptide modified AuNPs are shown. It can easily be seen that the data coming from the citrate capped and the peptide modified AuNPs are different. The intensities of the peaks in the SERS data differ from peptide to peptide. The peak intensities of the smallest and the largest anionic peptide modified AuNPs are low when compared to the others. This could be due to poor attachment to the Au surface due to the small or very large size of the peptides. For the longest anionic peptide, since there were too many negative charges on it, this might cause lower number of peptide binding to the surface resulting with lower intensity in peaks due to concentration effect. In Figure 5.5. B, the SERS data acquired from the citrate capped AuNPs and cationic peptide modified AuNPs are seen. Here it can also easily be seen that the SERS data acquired from the citrate capped and peptide modified AuNPs are different. The intensities of the peaks are very low when compared to the anionic peptide modified AuNPs. Also, the intensities are decreasing with the increase size of the peptide. Thus, it can be said that in longer-peptide-modified AuNPs, not many peptides were in close proximity to the NP surface.

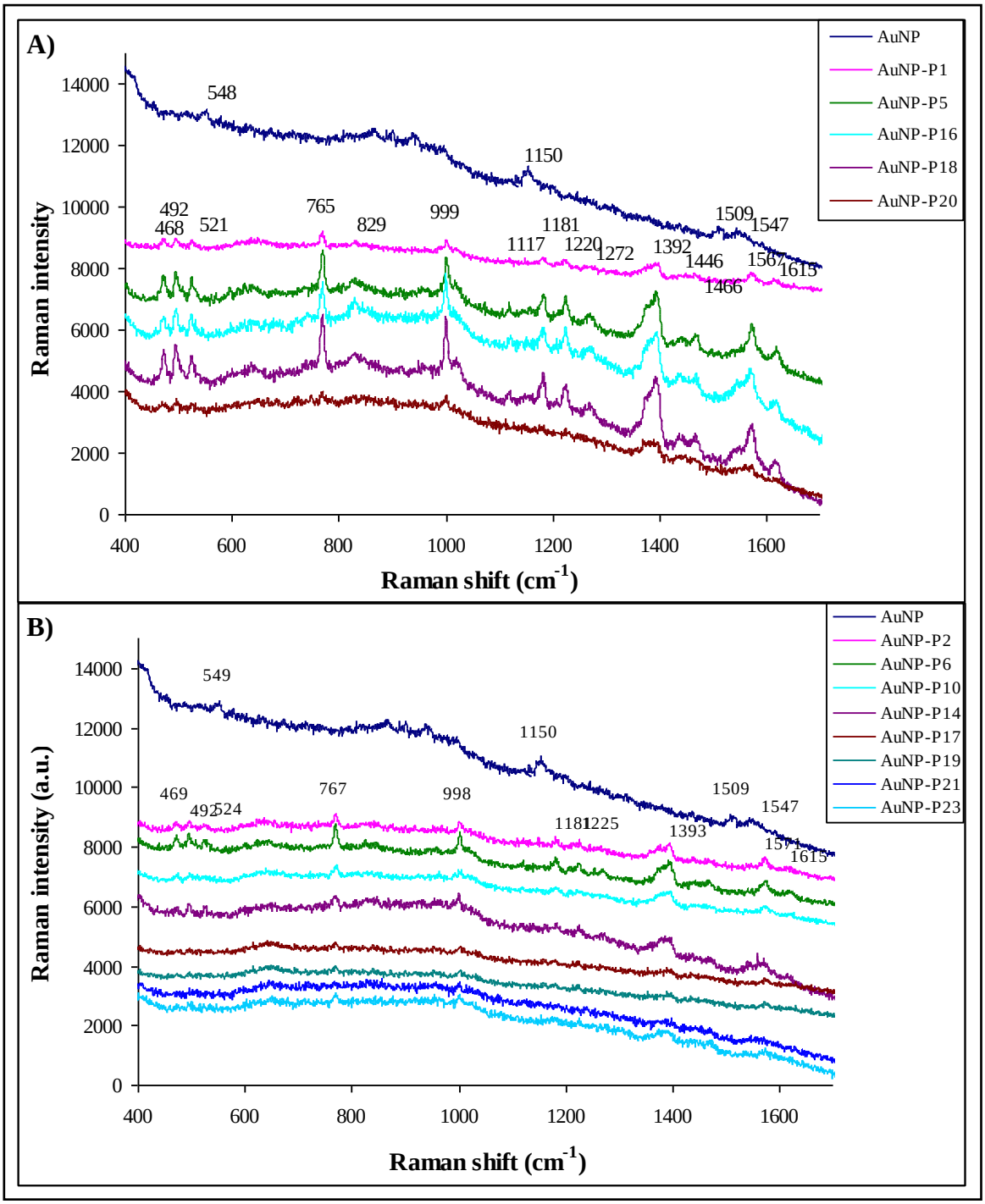

Figure 5.6. SERS of the unmodified and peptide modified AuNPs A) Anionic peptide modified B) Cationic peptide modified

5.2. BEHAVIOR of GOLD NANOPARTICLES in SUSPENSION and on SURFACES

It is critically important to understand the behavior of NPs in suspension and relate this information to their assembly on surfaces. Therefore, behavior of systematically modified AuNPs in suspension and interfaces were investigated. For this goal, in suspension, UV/Visible Spectroscopy, dynamic light scattering (DLS) and small angle X-Ray scattering (SAXS) were used. At interfaces, AFM was utilized.

5.2.1. Behavior of the AuNPs in the Suspension

The peptides used were divided into two groups according to their charge, namely anionic and cationic peptides. The sizes of the peptides also changes from 3 to 24 amino acids long. In theory, it was calculated that the shortest peptides were nearly 1 nm and longest ones were nearly 8 nm. In the study, the modified AuNPs were investigated according to their size and surface potential when modified with peptides. It was seen that anionic peptide modification caused nearly no size change. As can be seen in Figure 5.7, after the anionic peptide modification, nearly no change can be seen in UV/Visible spectra and also there is no change in their color. The maximum absorption of the all of the suspensions was 520 nm, which is the maximum absorption of the 13 nm AuNPs. The spectra acquired from the AuNP suspensions were narrow so it can be said that the size distribution is very uniform. It is also clear that no aggregates are formed in the suspension with the addition of peptides.

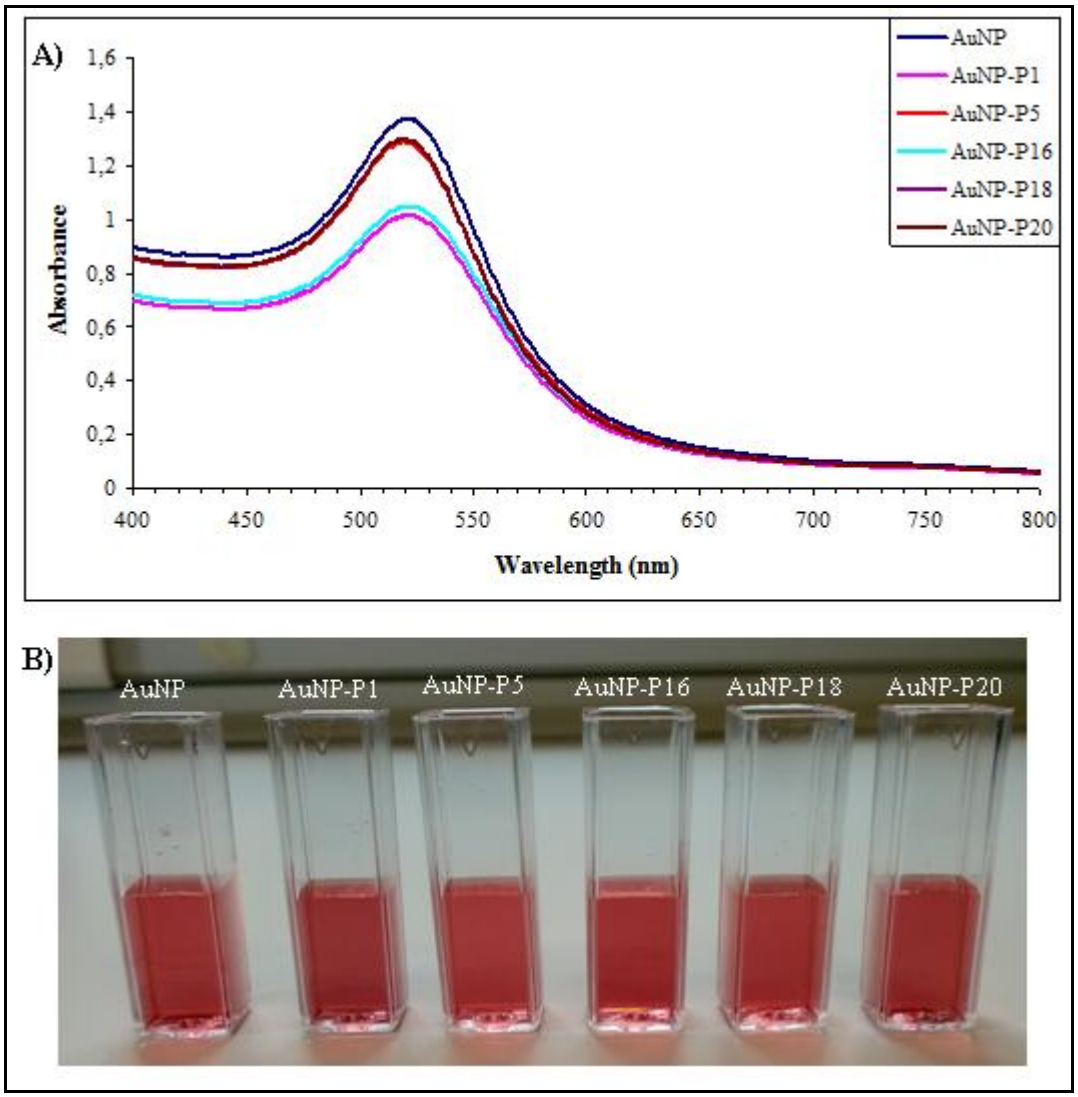

Figure 5.7. The UV/Vis spectra (A) and the photos of the AuNP suspensions (B) as they are systematically modified with negatively charged peptides

To get further information about the sizes of the peptide modified AuNPs and to observe whether there were aggregates or larger structures formed, intensity/size mode of the Zetasizer instrument was used. In the intensity/size mode, the bigger structures become more visible than the Number/size mode, due to the 10^6x multiplication of the scattered light. DLS measurements supported UV/Visible spectra. From the Figure 5.8 and Table 5.1, it can be said that there was no drastic change in the size of the AuNPs and no aggregates were observed after anionic peptide modification and also the surface potentials were nearly the same as citrate capped AuNPs although there was a very slight increased pattern in negativity of the peptide modified nanoparticles. As can be seen in Table 5.1, the

size of the citrate capped AuNPs were measured as 19.6 nm and the size of the longest anionic peptide modified AuNPs were 20,8 nm. This small increase might be due to the orientation of the peptides on the AuNP surface and steadiness of the surface charge after the peptide citrate exchange. The surface potential of the citrate capped AuNPs was -31 mV whereas the surface potential of the longest peptide modified AuNP was -38 mV. The surface potentials of the other peptide modified AuNPs were between these two values. Thus, it was seen that before and after the peptide modification of the AuNPs, the behavior of the AuNPs did not change in suspension. A very small size change was observed and only 7 mV differences was measured between the citrate capped AuNPs and 21 amino acid long peptide modified AuNPs.

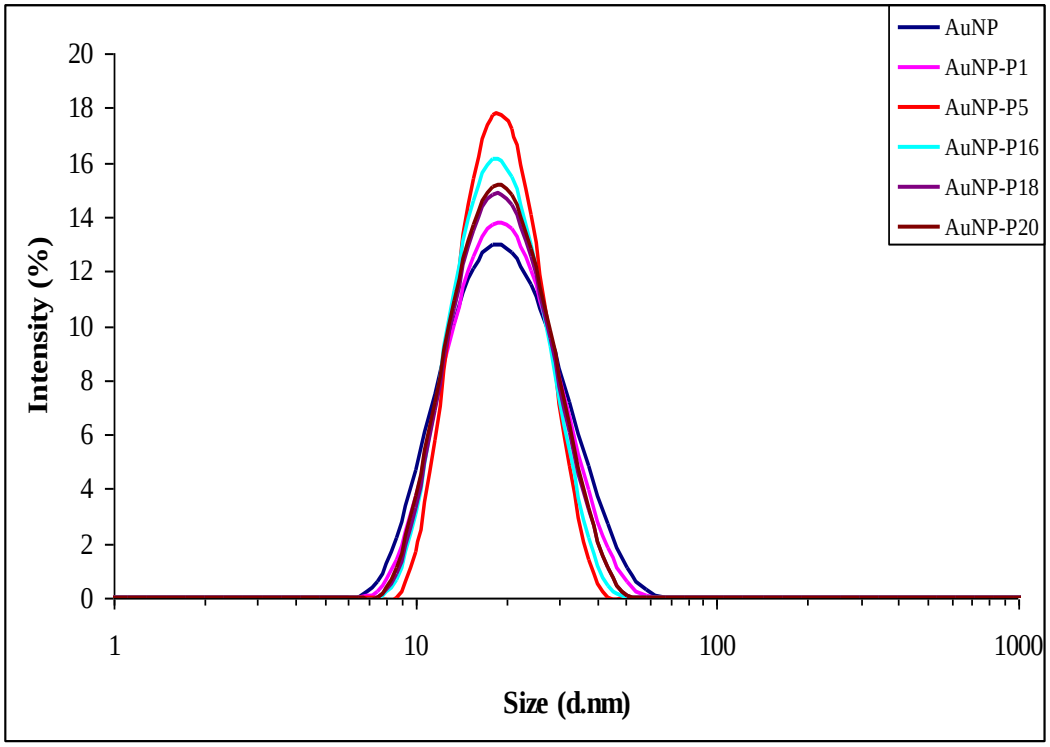

Figure 5.8. The hydrodynamic radii of the AuNPs modified with anionic peptides.

Table 5.1. The hydrodynamic radii and their zeta potentials of the AuNPs modified with anionic peptides.

Measured AuNPs	**Sizes (nm)**	**Zeta potential (mV)**
AuNP	19,6 nm	-31 mV
AuNP-P1	20,1 nm	-35 mV
AuNP-P5	19,7nm	-35 mV
AuNP-P16	19,8 nm	-36 mV
AuNP-P18	20,0 nm	-36 mV
AuNP-P20	20,8 nm	-38 mV

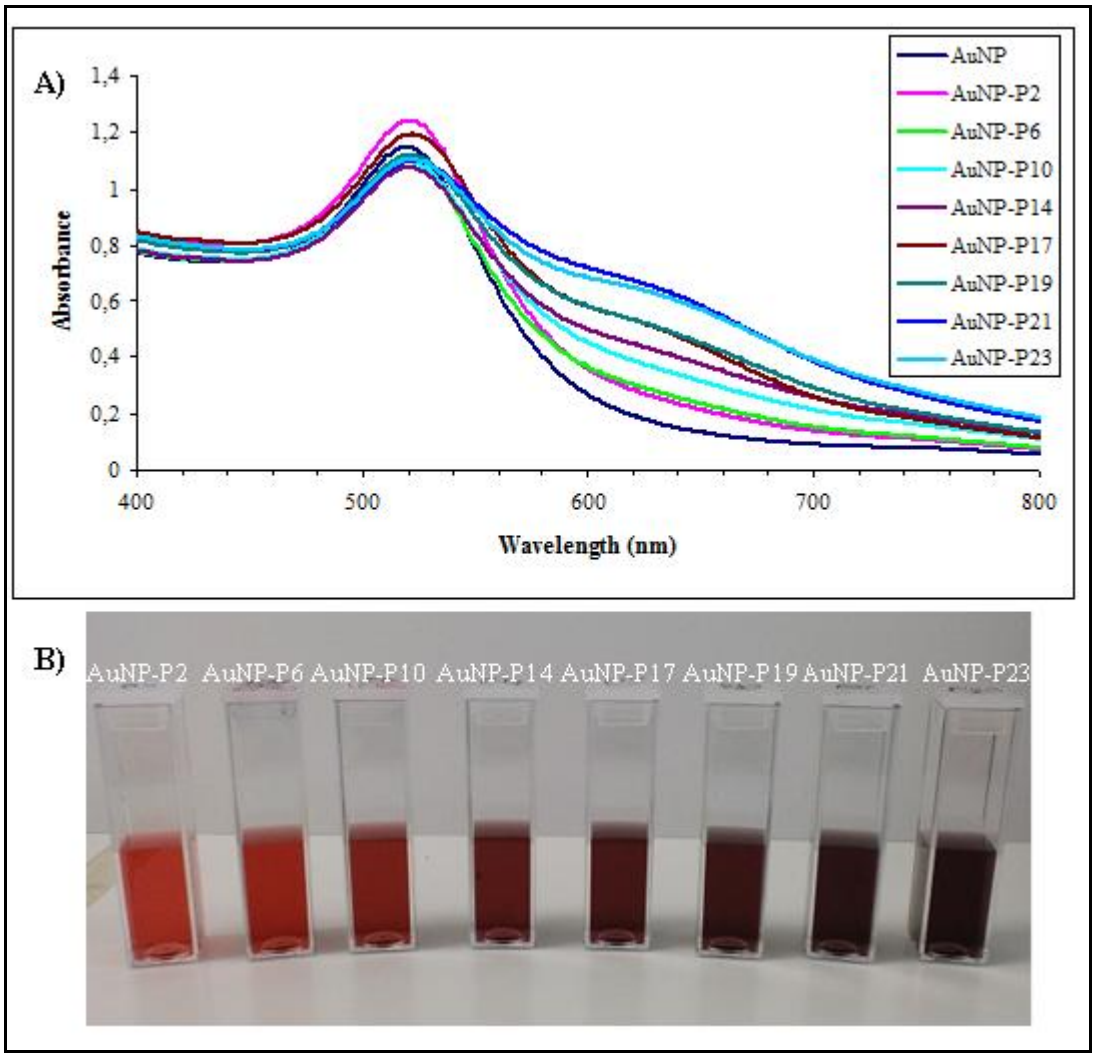

Figure 5.9. The UV/Vis spectra (A) and the photos of the AuNP suspensions (B) as they are systematically modified with negatively charged peptides

On the UV/Vis spectra of AuNPs modified with positively charged peptides, the peak at 520 nm remained unchanged after cationic peptide modification. However, as the size of peptides increases, another peak at around 630 nm started to emerge. Meanwhile, a decrease in the intensity of the peak at 520 nm was observed indicating that some of modified AuNPs change their aggregation status. As seen from 5.9B, the color of the suspension start to change from ruby red to dark blue which was the indicator of the size increase due to the change in oscillation of the surface electrons. There are two possible explanation of this pattern. First one is the formation of random aggregates. There are both aggregates and individual 13 nm AuNPs in the suspension. The second possible

explanation is that ~630 nm shoulder and 520 nm peak were seen as result of the nanowire formation in the suspension [105].

To get more information about structures formed in suspension, Zetasizer measurements were used. Both the sizes of the structures and the surface potentials were investigated. When the DLS data is interpreted, it can be seen that the sizes increased with the increased length of peptides. From Figure 5.10, the size of the citrate capped AuNPs were 19,6 nm, the average size of the P2 modified AuNPs are ~33 nm and when the AuNPs were modified with P23, which is the 24 amino acid long peptide, the size of the nanostructures becomes ~60 nm. Details can be seen in Table 5.2. The size increase is thought to be the result of the interaction of the positive amine groups with the negative citrates found on the surface of the AuNPs. The surface of the citrate reduced AuNPs have citrate ions which give the AuNPs a negative charge and stabilize them in suspension. After addition of the cationic peptides, the peptides containing affinity groups exchange with the citrate on the surface of the AuNPs. In the case of cationic peptides, the citrate was thought to act as a cross linker between positive parts of the cationic peptide modified AuNPs. This electrostatic interaction, in principle, should have been insignificant and reversible, but the cumulative effect of individual interactions may cause a high attraction force, causing immediate aggregation. These citrates could not be removed by using dialysis due to these attraction forces. There can also be some problems in binding of the cationic peptides. It is known that as well as the –SH groups, –NH groups also have an affinity to bind to the Au surface. The $–NH_2$ groups coming from the side chains of the cationic amino acids can interact with the gold surface which reduces the number of peptides that can bind to the AuNPs. This can also affect the surface charge.

When the surface potentials of the cationic peptide modified AuNPs were investigated, cationic peptide addition did not have a positive charge effect. Details can be seen in Table 5.2. The citrate capped AuNPs had a surface potential of -31 mV whereas the most cationic peptide modified AuNPs had a surface potential of -35 mV. Since the citrates could not be removed from the suspension, this could cause this surface potential instability. But there could be measurement problems in the DLS. The DLS instrument assumes that the particles measured are spherical. But cationic peptide modified AuNPs might not be spherical due to the interaction between the citrate and amine groups. These

non spherical structures might not be measured properly. Thus, the sizes of the cationic peptide modified AuNPs and surface charges might be different than the measured ones.

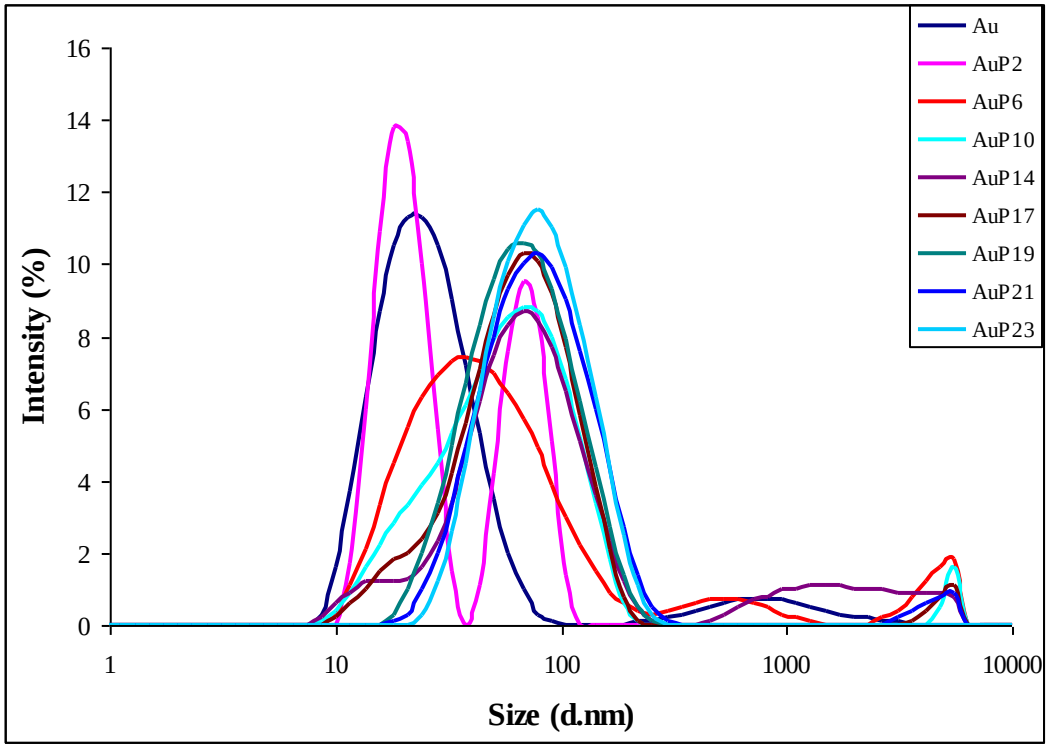

Figure 5.10. The hydrodynamic radii of the AuNPs modified with cationic peptides.

Table 5.2. The hydrodynamic radii and zeta potentials of AuNPs modified with cationic peptides.

Measured AuNPs	Sizes (nm)	Zeta potantial (mV)
AuNP	19,6 nm	-31 mV
AuNP-P2	33,7 nm	-35 mV
AuNP-P6	41,8 nm	-34 mV
AuNP-P10	50,3 nm	-36 mV
AuNP-P14	55,2 nm	-30 mV
AuNP-P17	54,4 nm	-29 mV
AuNP-P19	54,9 nm	-34 mV
AuNP-P21	62,5 nm	-37 mV
AuNP-P23	65,2 nm	-35 mV

5.2.2. Behavior of the AuNPs on the Surfaces

The behavior of the peptide modified AuNPs on the surfaces after drying and their structural transferability from suspension to surfaces was studied. The peptide-modified AuNP containing suspensions were placed as a droplet on hydrophilic surfaces and their assembly was investigated by using AFM and SEM. As a hydrophilic surface, mika, which has a very smooth surface, is used.

From the AFM images of Figure 5.13 and 5.14, it was seen that there was no indication of assembly of negatively charged peptide modified AuNPs and no difference between the citrate capped and anionic peptide modified AuNPs. The nanoparticles are distributed randomly. It is most probably due to the excess negative charge on the nanoparticle surfaces; the nanoparticles cannot form networks or large aggregates.

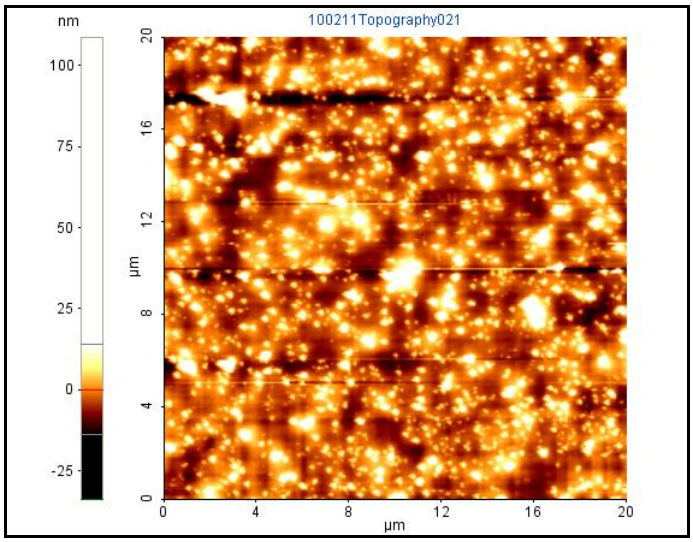

Figure 5.11. AFM image of the citrate capped AuNPs

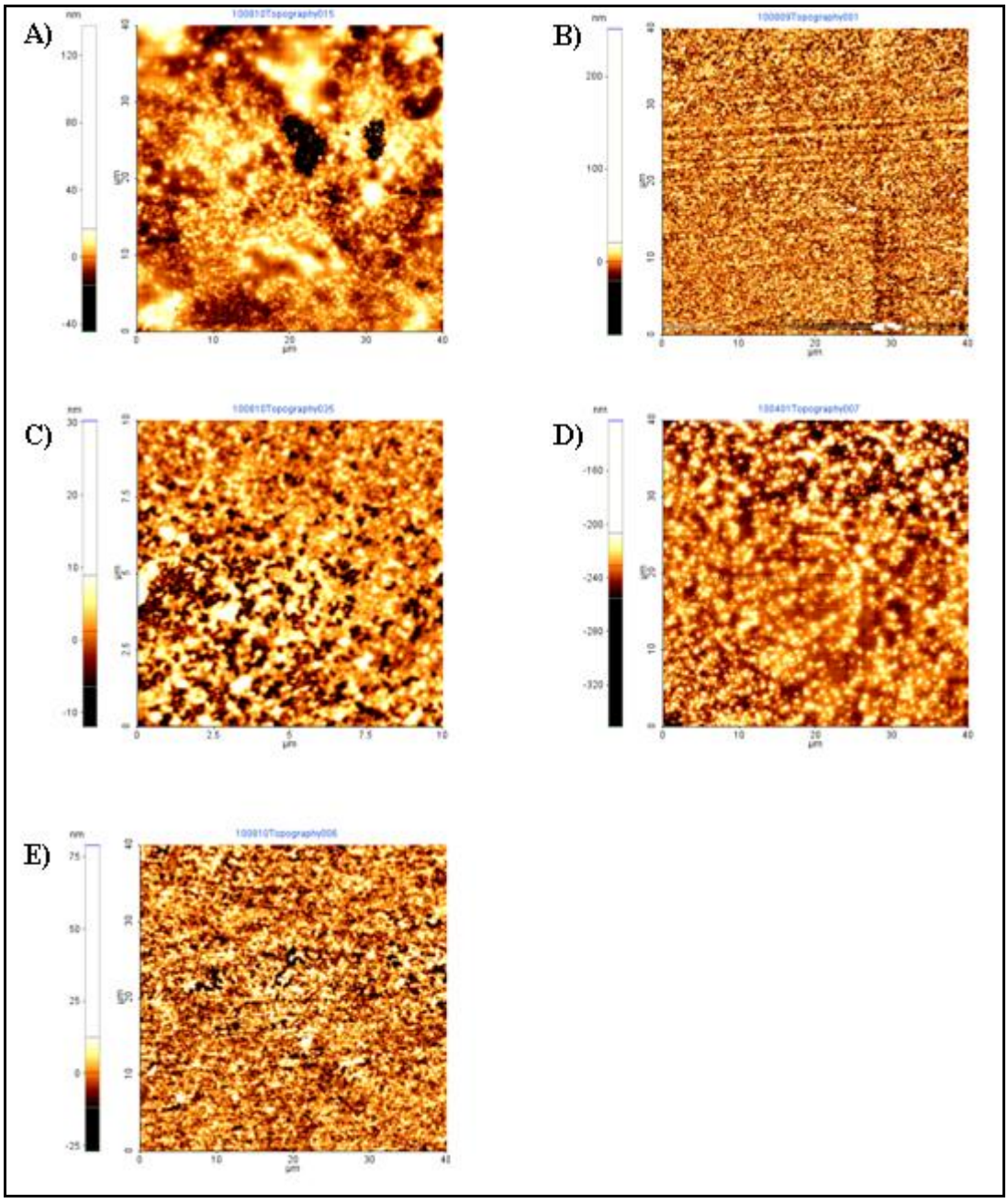

Figure 5.12. AFM images of the anionic peptide modified AuNPs A) P1 modified AuNPs B) P5 modified AuNPs C) P16 modified AuNPs D) P18 modified AuNPs E) P20 modified AuNPs

Figure 5.15 shows the AFM images of structures formed from a drying droplet of positively charged peptide modified AuNPs on the surfaces. As seen in Figure 5.15B and C, as the size and charge on the peptide increases, the formation of larger structures are visible. As the size of peptide increases further, the formation of rod –like structures are

formed and can be seen in Figure 5.15 D-H. These observations are consistent with UV/Vis and DLS data.

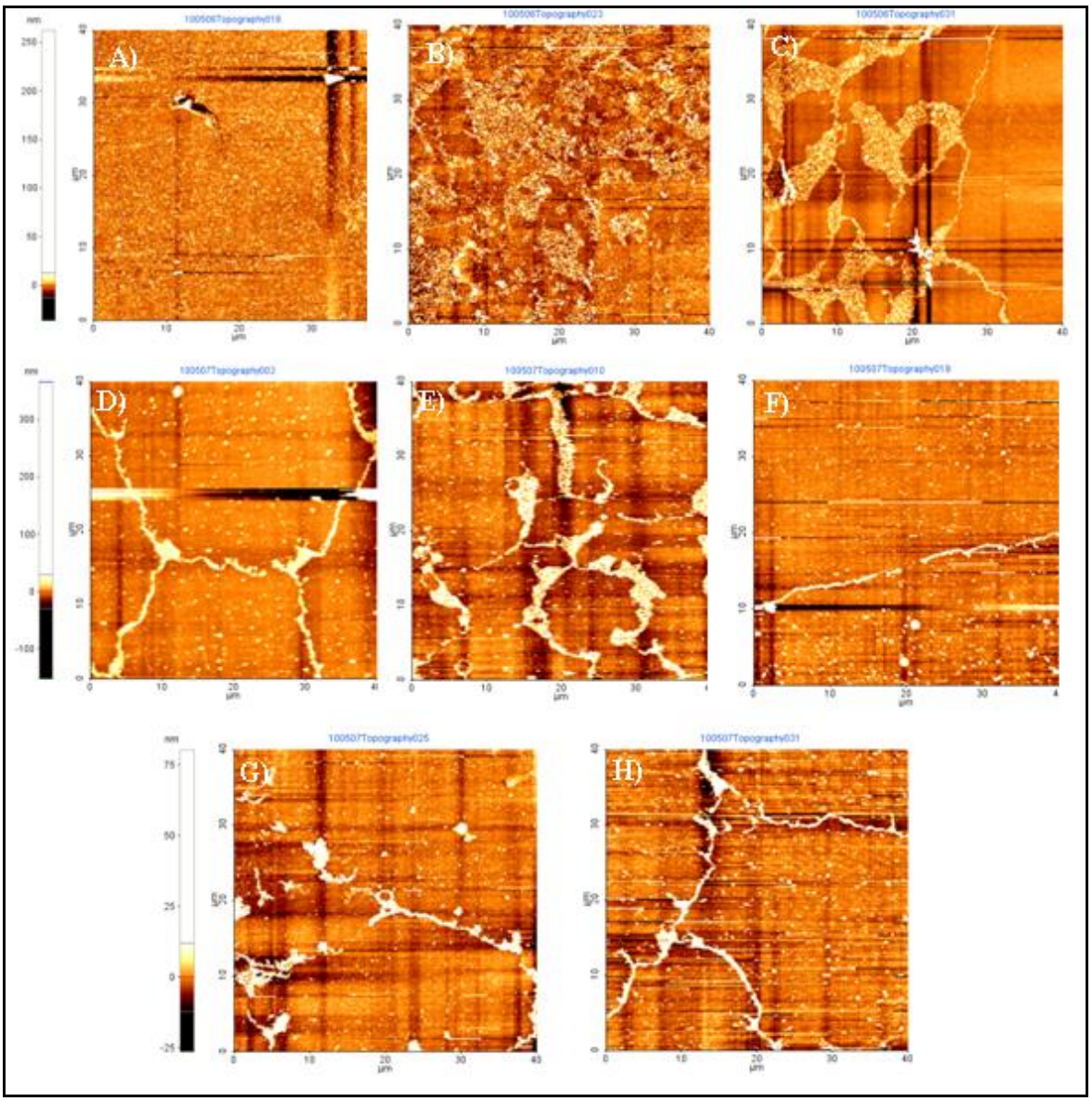

Figure 5.13. AFM images of the cationic peptide modified AuNPs A) P2 modified AuNPs B) P6 modified AuNPs C) P10 modified AuNPs D) P14 modified AuNPs E) P17 modified AuNPs F) P19 modified AuNPs G) P21 modified AuNPs H) P23 modified AuNPs

When the structures formed in the surfaces investigated more closely, in Figure 5.14, it can be seen that the width of the wire is nearly 500 nm and it has a height of one or two

NPs. This indicates that the structures formed are a few nanoparticle thick 35-40 nanoparticle wide flat structures

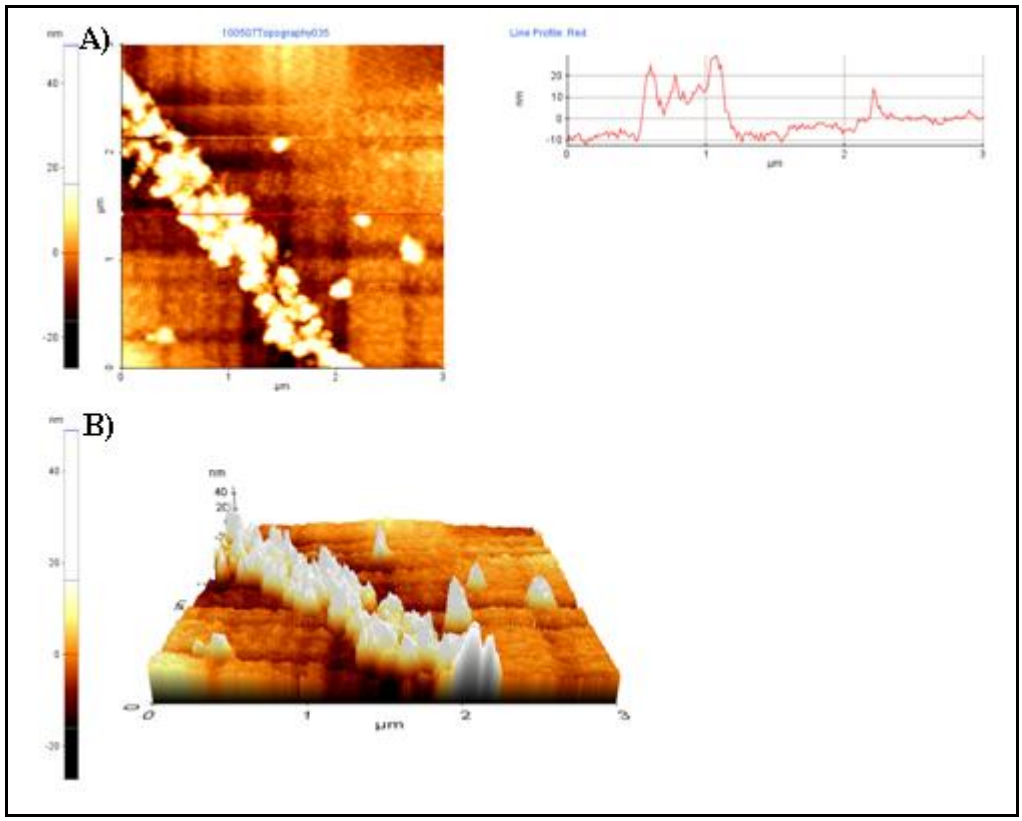

Figure 5.14. AFM image of the P23 modfied AuNPs A) AFM image and the line analysis of it B) 3-D view of the A

The formation of extended network-like structures can be attributed to the formation of salt-bridges between the amino groups of peptides and free C-terminus of the peptides attached to the AuNPs through –S-Au or –N-Au bonds. Since the number of $–NH_2$ groups increases with the size of the peptide, these side amino groups seem to play important role in the formation of the observed structures.

6. CONCLUSION AND RECOMMENDATION

6.1. CONCLUSION

In this study, we aimed to understand the behavior of the peptide modified AuNPs. The AuNPs were used as model nanoparticles due to their plasmonic properties, fairly uniform size synthesis, and well-defined and easy surface chemistry. The behavior of the peptide treated AuNPs examined in their suspension and the transferability of these behaviors on surfaces were investigated. The 13 different peptides, 5 of them were anionic and 8 of them were cationic peptides were investigated.

For the anionic peptide modified AuNPs, in suspension, no color change was observed after peptide modification and the UV/Visible spectra were the same with citrate-capped AuNPs. From zetasizer measurements, very small change in aggregate size and surface potential were observed. Also nearly no difference was observed between the anionic peptide and citrate capped AuNPs.

For the cationic peptide AuNPs, in the suspension part, the colors of the colloidal suspensions changes from ruby red to dark bluish color with the increasing size of the peptides. In their UV/Visible spectra, a sharp 520 nm band was observed and with the peptide size increase, a ~620 nm shoulder started to emerge. It is observed that the surface potential was not related to bound peptides. The sizes increase with the increased length of the bound peptide. In the surface, wire like structures were observed and sizes of these wires increased with the increased positive charge of the bound peptide.

In conclusion, it was found that charge of the peptides have a dominating effect on the assembly of peptide modified AuNPs AuNPs and they can be utilized to influence the behavior of nanoparticles for manipulation to bring them to higher structures

6.2. RECOMMENDATION

The assembly of nanostructures from smaller building blocks such as nanoparticles is an emerging area of nanoscience and nanotechnology. As the new routes are explored for the goal, it will be possible to construct novel tools that will enhance our lives. The use of interactions such as electrostatic and hydrophobic that govern the processes living systems may introduce new breath to this field. In this extend, the use of biological macromolecules seems inevitable, which the pronounced forces are already available through them. Proteins and peptides are the major structural bricks in biological systems due to their chemical, conformational and functional diversity. As a future work of this project, by using these properties, different peptides can be designed. Coiled coil peptides are one of the peptides that can be designed to use for self assembly. Also, peptides which assemble to nanotubes can be used to assemble the nanoparticles inside or outside of the tube. The major interaction mode investigated in this study was electrostatic. The assembly process with introduction of hydrophobic interactions along with electrostatic interactions can be investigated as an extension of this study. The use of molecular modeling can help to design and investigate behavior of the modified AuNPs to generate higher organizations.

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