A PLASMONIC METHOD FOR THE DETERMINATION OF SEROTONIN

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ABSTRACT

A PLASMONIC METHOD FOR THE DETERMINATION OF SEROTONIN

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Serotonin is an important neurotransmitter for regulating many cognitive and behavioral functions. Its abnormal levels have been related with various diseases hence the determination of the serotonin is very important. Sialic acid is mostly found in acidic glycan chains, glycolipids and glycoproteins and it is known to have a specific affinity for serotonin.

In this study, determination of serotonin by using plasmonic property of gold nanoparticles was aimed. After synthesizing gold nanoparticles through sialic acid reduction, the method was simply based on the red shift in absorbance peak of gold nanoparticles at 520 nm upon aggregation of gold nanoparticles which was induced by the reaction between serotonin and sialic acid. This shift was seen only for serotonin among serotonin, dopamine, *l*-cysteine, *l*-aspartic acid and *l*-glutamic acid and it has shown that the determination of serotonin can be performed in a simple and selective way by measuring the absorbance with an Ultraviolet-Visible (UV-VIS) spectrophotometer. The method successfully worked for serotonin concentration between 10 and 500 μM. To be able to work with serotonin levels in blood between 0.5 and 1.7 μM some modifications on gold nanoparticles were performed. Making selective side of sialic acid available for serotonin binding was aimed by attaching carboxyl group of sialic acid onto amine group containing gold nanoparticles so that

an enhancement in aggregation can be achieved. The studies on binding sialic acid to gold nanoparticles from its nonselective side for serotonin are in progress.

Keywords: Gold Nanoparticles, Sialic Acid, Serotonin, Colorimetric Determination

ÖZ

SEROTONİN TAYİNİ İÇİN PLAZMONİK BİR YÖNTEM

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Serotonin, birçok bilişsel ve davranışsal işlevin düzenlenmesi için önemli bir nörotransmitterdir. Olağandışı serotonin seviyeleri çeşitli hastalıklarla ilişkilendirildiği için tayini çok önemlidir. Sialik asit çoğunlukla asidik glikan zincirlerinde, glikolipidlerde ve glikoproteinlerde bulunur ve serotonine özel ilgisi olduğu bilinmektedir.

Altın nanoparçacıklar, sialic asidin indirgen özelliği kullanılarak sentezlendikten sonra, yöntem basitçe, serotonin ve sialik asit arasındaki reaksiyona bağlı olarak altın nanoparçacıkların topaklaşması üzerine altın nanoparçacıkların 520 nm'deki absorbans pikinin uzun dalga boylarına kaymasına dayanmaktadır. Serotonin, dopamin, *l*-sistein, *l*-aspartik asit ve *l*-glutamik asit arasında sadece serotonin için görülen bu kayma, Mor Ötesi-Görünür bölge (UV-VIS) spektrofotometresi ile absorbans ölçerek, serotonin tayinin basit ve seçici bir şekilde yapılabileceğini göstermiştir. Yöntem 10 ila 500 µM arasındaki serotonin derişimleri için başarıyla çalışmıştır. Kandaki serotonin düzeyleriyle (0,5 ila 1,7 µM arasında) çalışabilmek için altın nanoparçacılar üzerinde modifikasyonlar yapılmıştır. Serotonin bağlanması için sialik asidin seçici tarafının uygun hale getirilmesi amacıyla sialik asidin karboksil grubunun, amin grubu içeren altın nanoparçacıklara bağlanması ve böylece

topaklaşmada bir artışın elde edilmesi amaçlanmıştır. Sialik asidi, altın nanoparçacıklara seçici olmayan tarafından bağlama çalışmaları devam etmektedir.

Anahtar Kelimeler: Altın Nanoparçacıklar, Sialik Asit, Serotonin, Kolorimetrik Tayin

To My Beloved Family

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LIST OF ABBREVIATIONS

ABBREVIATIONS	
AuNPs	Gold nanoparticles
SA-AuNPs	Sialic acid stabilized gold nanoparticles
MBTH	3-Methyl-2-benzothiazolone hydrazone
UV-VIS	Ultraviolet-Visible
SEM	Scanning electron microscope
LOD	Limit of detection
LOQ	Limit of quantitation
f-AuNPs	Functionalized gold nanoparticles
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDC+SA-AuNP	EDC added sialic acid stabilized gold nanoparticles
NHS	N-Hydroxysuccinimide
AuNPs-L-cys+EDC/NHS	EDC and NHS added, L-cys coated gold nanoparticles
AuNPs-NH_3^+	Cysteamine coated gold nanoparticles
SA+EDC/NHS	EDC and NHS added sialic acid
TEA	Triethylamine

CHAPTER 1

INTRODUCTION

1.1. An Introduction to Nanotechnology

Nanotechnology has become one of the most popular fields of science after development of many methods for synthesizing and characterizing materials at nanoscale [1]. Nanotechnology involves methods to design, synthesize, control, characterize and apply materials or devices whose at least one dimension is at the scale of one billionth of a meter [2]. As being an interdisciplinary field of science, nanotechnology is used in chemistry, biology, medicine, engineering and it is expanding limits of research [3]. The increasing progress in nanotechnology meets the demands of studies in these fields [4].

In chemistry, nanoparticles either inorganic or organic are synthesized and used for their specific property or composition and they are usually smaller than 100 nm in size. When describing nanoparticles, all nanoparticles synthesized from metals, oxides, semiconductors as well as from nontraditional and nonmetal ones are included [5].

1.1.1. Plasmonic Nanoparticles

When metal nanoparticles interact with electromagnetic radiation, surface conduction electrons oscillate collectively and this phenomenon is called surface plasmon. An enhanced magnetic field on the metals' surface was produced by surface plasmons after interacting with electromagnetic radiation [6]. The reason of referring 'surface plasmon' is that the ability of penetration of an electromagnetic wave is limited with the surface. In addition, if the oscillation of surface electrons is confined with a finite volume, the resulting plasmon is named as localized surface plasmon. Figure 1.1. shows the interaction between surface electrons of a metal and incident light at a

specific wavelength. The electric field causes the movement of surface electrons in one direction and creates a dipole which can change its direction upon electric field. If this dipole plasmon's frequency approximately equals to incident light's, localized surface plasmon resonance (LSPR) is produced [7].

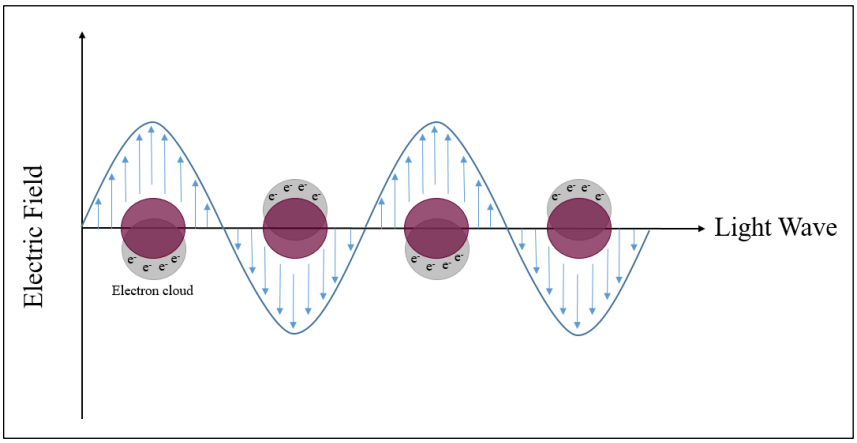


Figure 1.1. LSPR of a metal nanoparticle after interacting with an incident beam [7]

The amplitude and frequency of resonance depend on the size, shape and aggregation state of nanoparticles proved by measurements as well as calculations [7]. The ability of designing these properties is the most powerful feature of plasmonic nanoparticles.

Noble metal nanoparticles are highly advantageous due to their chemical inertness and their nature of having localized surface plasmon resonance in the visible or near-IR regions [8]. As an example, the intensive color of gold nanoparticles under illumination is one of the reasons why they have been popular since ancient times [6].

1.2. Gold Nanoparticles

Gold nanoparticles (AuNPs) show unique and distinguishing chemical and physical properties than gold atoms and bulk gold. These properties can be adjusted by changing AuNPs' size, shape, aggregation state and dielectric environment [9, 10]. This is why AuNPs have been drawing great interest in many fields including

catalysis, electronics, biosensing, constructing solar cells, drug delivery and others [4], [11].

1.2.1. Applications of Gold Nanoparticles

In electrochemistry, AuNPs are widely used due to their high surface to volume ratio, high conducting capability and biological compatibility [11].

One of the biggest application areas of AuNPs is medicine [3] since they are inert and usually nontoxic. After synthesizing the particles at nanoscale, they can be functionalized due to targeted molecule [12] and used for drug delivery.

A wide variety of applications of AuNPs exists due to their unique plasmonic property. Plasmonic property of gold nanoparticles can be used for enhancing the Raman scattering of molecules adsorbed on the surface of gold which let to development of surface enhanced Raman scattering (SERS) spectroscopy [13, 14]. For instance, Gu et al. [15] reported a plasmonic sensor of gold nanoparticles modified with 3-mercaptophenylboronic acid (3-MPBA) for H_2O_2 sensing.

In terms of optical sensing, peak position and the intensity of the LSPR peak enable to monitor binding ability of AuNPs. Attaching a biological ligand on to surface of AuNPs [15, 16], therefore, provides detection strategies for biomolecules in a very sensitive and low cost way. Riedel et al. [18] developed a biosensor by adsorption of poly[(N-(2-hydroxypropyl)methacrylamide)-co-(carboxybetaine methacrylamide)] onto gold surface to detect hepatitis B in saliva with a fluorimetric method. Hiep et al. [19] presented an interference localized surface plasmon resonance (iLSPR) sensor depending on gold nanoparticles whose surface was covered by multilayers of porous aluminum oxide (Al_2O_3) and aluminum (Al) on a substrate so that changes in refractive index can be monitored to detect biotin and avidin after functionalizing the surface of iLSPR. In another method developed by Huang et al. [20] a plasmonic sensor of gold nanorods was used to detect Fe^{3+}, Hg^{2+}, Cu^{2+} and Ag^{+} ions causing the change in nanostructure monitored by the shift in longitudinal plasmon wavelength (LPW) of nanorods under proper conditions.

1.2.2. Synthesis of Gold Nanoparticles

The first scientific report on synthesis of colloidal AuNPs belongs to Michael Faraday. He reported the 'fine particles' produced from the reduction of gold chloride by phosphorous and described the resulting solution as 'beautiful ruby fluid' in 1857 [21]. After discovering that the shape and size of AuNPs distinctively affect the interaction with electromagnetic radiation and thus LSPR, scientists have been developing methods to synthesize gold nanoparticles with different morphologies [7]. Most of these methods are based on reduction of solvated gold salts by surface capping ligands so that the aggregation of AuNPs can be prevented by electrostatic repulsion [21].

Turkevich's method based on citrate reduction [22] and reduction by sodium borohydride have been one of the oldest methods to synthesize AuNPs. While reduction by sodium citrate produces monodisperse, spherical AuNPs, that by sodium borohydride provides synthesis with high concentration of AuNPs [10]. Ascorbic acid is also one the popular reducing agents [23].

Even though gold spheres were the basis of the early studies, various types of AuNPs have been synthesized with different sizes and shapes [3] including gold nanorods [24], nanocages [25] and nanoshells [26].

1.2.2.1. Synthesis of Sialic Acid Stabilized Gold Nanoparticles

Sialic acid represents the family of nine-carbon sugar neuraminic acid derivatives. The amino group and the carboxyl group having negative charge on it under physiological conditions make neuraminic acid a strong acid. In nature, an unsubstituted form of neuraminic acid does not exist. The most common form of sialic acid is the one whose amino group is acetylated, called N-acetylneuraminic acid (Neu5Ac) [27]. The chemical structure of N-acetylneuraminic acid is shown in Figure 1.2.

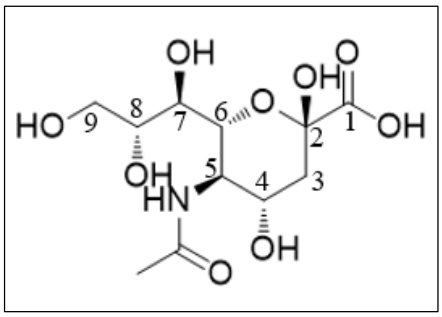


Figure 1.2. Chemical structure of N-acetylneuraminic acid (sialic acid)

The family of sialic acids, N-acetylneuraminic acid and its derivatives, are the terminal units of many glycan chains [28]. Due to their negative charge and hydrophilicity, sialic acids have been correlated with wide variety of biological functions. They present a specific binding side for many toxins and pathogens [29].

Lee et al. [30], designed a method to detect a type of an influenza virus by using binding side of sialic acid for detection. To achieve this, they synthesized sialic acid stabilized gold nanoparticles (SA-AuNPs) having approximately 20 nm size by using reducing ability of sialic acid. They reported that all five hydroxyl groups and one N-acetyl group have the ability of reducing gold salt to form SA-AuNPs.

1.3. Methods for Sialic Acid Determination

Many methods have been developed to determine sialic acid. Most of these methods rely on color formation after reaction with sialic acids. Hess et al. [31] suggested a method based on reaction of sialic acid with diphenylamine and the resulting product was colored. In addition, Svennerholm [32] developed resorcinol method for sialic acid determination by measuring the absorbance of colored product of sialic acid with Cu^{2+} and Fe^{3+} ions. Although these methods offer effective measurement, they require heating step with strong acids which makes difficult to distinguish between the free and bound sialic acid impossible [33].

To estimate free sialic acid, Paerels and Schut [34] came up with a new method depending on chromogen formation after sialic acid and periodate thiobarbituric acid reaction.

For the sialic acid residues in glycoproteins, a fluorimetric method in which sialic acid was oxidized by periodate and the formaldehyde formed from 9^{th} carbon (C-9) of sialic acid converted to a fluorescent molecule was developed by Matsuno and Suzuki [35].

By periodate oxidation of sialic acid, the resulting formaldehyde formed from cleavage of C-8 and C-9 is stoichiometric mole to mole with sialic acid. Massamiri et al. [36] developed a colorimetric method for sialic acid determination by using the reaction between methyl-3-benzothiazolinone-2-hydrazone (MBTH) molecule and the formaldehyde cleaved from sialic acid.

In recent years, the number of methods increased for the determination of sialic acid. Costa et al. [37] came up with a method to determine sialic acid in milk sample based on a flow-batch system analysis after reaction with acid ninhydrin and the resulting product had maximum absorption at 470 nm followed by the spectrophotometric measurement. On the other hand, Jayeoye et al. [38] reported a sensitive probe of gold nanoparticles functionalized with dithiobis(succinimidylpropionate) (DSP). After amine-carboxyl coupling of nanoparticles with 3-aminophenyl boronic acid (3APBA), aggregation of modified nanoparticles induced by sialic acid was monitored by a spectrophotometer. An electrochemical determination method was developed by Zheng et al. [39] which was based on a capillary electrophoresis with amperometric detection system. After oxidation of sialic acid (NANA) to ß-formyl pyruvic acid, an electroactive NANA-TBA was formed from reaction of electroactive 2-thiobarbituric acid (TBA) with ß-formyl pyruvic acid to measure with the electrophoresis device.

1.3.1. The 3-Methyl-2-benzothiazolone Hydrazone (MBTH) Method

The usefulness of MBTH molecule for colorimetric determination of sialic acid was first reported by Massamiri et al. [40]. The MBTH method involved two steps. The

first step was the condensation reaction of formaldehyde formed from the oxidation of sialic acid by periodate with hydrazone group of MBTH. Second step was the reaction between product of former reaction and the oxidation product of excess MBTH by Fe^{3+} ions resulting in a blue colored cationic dye which absorbs light at 625 nm. Grevel and Mutus [41] also determined sialic acid by oxidizing it to form formaldehyde by modifying the method developed by Massamiri et al.

Honda et al. [42] reported a method based on the condensation reaction of a carbonyl group with the hydrazone group of MBTH molecule under alkaline medium and the resulting product had λ_{max} at 390 nm. This group showed that sugars can be determined by this method under alkaline conditions at elevated temperatures by spectrophotometry. They reported only sensitivity of sialic acid relative to D-glucose given as molar absorptivity of sialic acid was 7 times higher than the one of D-glucose.

In 2002, Anthon and Barret [43] developed a procedure to determine reducing sugars by modifying MBTH method without former formation of aldehyde from reducing sugars. Although sialic acid is a family of nine-carbon sugar, there is no published study on determining sialic acid by MBTH method without modification of sialic acid. The application of Anthon and Barret's procedure on SA-AuNPs to determine the amount of sialic acid on the surface of nanoparticles without former oxidation step of sialic acid will be explained in the experimental chapter.

1.4. Serotonin

Serotonin (5-hydroxytryptamine) is a small and important molecule having two functions in human body: a neurotransmitter in the central nervous system and a hormone in the periphery. Serotonin is synthesized after a series of reactions in the body. First, L-tryptophan is converted into 5-hydroxy-L-tryptophan by tryptophan hydroxylase (Tph) enzyme and then, 5-hydroxy-L-tryptophan is converted to serotonin by an aromatic L-amino acid decarboxylase [44] as shown in Figure 1.3. Serotonin is a monoamine type [45] of neurotransmitter.

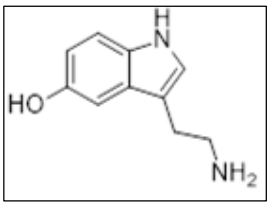


Figure 1.3. Chemical structure of serotonin

Serotonin is produced in brain and gastrointestinal tract and these two pools of serotonin never mix since it does not cross the brain-blood barrier [44]. Serotonin has a very important role in regulating many behavioral and cognitive functions like mood, sleep, appetite, learning, pain, sexuality and cardiovascular function [36–39].

High and low levels of serotonin in blood have been related with number of diseases. Its low levels have been associated with depression, anxiety, migraines, attention deficit hyperactivity disorder (ADHD), autism and inflammatory syndromes [49], whereas high levels associated with toxicity and serotonin syndrome [46]. As a result, several methods have been developed to determine serotonin which will be discussed in the next section.

1.4.1. Methods for Serotonin Determination

Serotonin levels in human blood is between 0.5 μM and 1.7 μM for healthy people [50]. For serotonin determination, liquid chromatography [36, 42–44], electrochemical techniques [45–48], phosphorescence [58] and fluorimetric [59] methods have been developed. Although these methods can successfully measure low serotonin levels, most of them require high cost equipment. Additionally, they are time consuming particularly liquid chromatography.

Developing a method for the determination of serotonin is challenging since the molecule is complex and there are many interferences may come from the environment of the serotonin molecule that resemble it [49].

In recent years, developing chromogenic probes for detection of biomolecules has become popular. Especially, using gold nanoparticles attracts attention since they are relatively cost friendly, easy to use and have excellent plasmonic properties [6]. Godoy-Reyes et al. [49] developed a probe by functionalizing gold nanoparticles selective to serotonin. Two types of molecules were used for attaching nanoparticles to serotonin; dithiobis(succinimidylpropionate) (DSP) from primary amine side and N-acetyl-l-cysteine (NALC) from hydroxyl group. Thus, aggregation of functionalized gold nanoparticles by addition of serotonin was aimed. As a result, the change in plasmonic properties of particles was monitored by absorbance measurement and the red shift of the absorbance spectrum was related with the concentration of serotonin. Limit of detection (LOD) for serotonin with this method was reported as 0.1 μM. Another spectrophotometric method for determination of serotonin derivatives was reported by Jin et al. [60]. The method was based on the formation of a colored product formed from reaction of serotonin derivatives with and *p*-dimethylaminobenzaldehyde having absorbance at 625 nm. The disadvantage of the method was that it was not selective to serotonin and LOD for serotonin derivatives was determined as 25 μM.

By considering the chemical structure and properties of the serotonin, various types of chromogenic probes can be designed.

1.4.2. Serotonin Affinity to Sialic Acid

As it has been mentioned before, sialic acids involve in many processes of glycans, glycoproteins, glycolipids and they have recognition for many biomolecules as they form specific binding sides [51, 52]. One of these biologic entities is serotonin. The affinity of sialyted glycoproteins for serotonin was first reported by Ochoa and Bangham [62]. Sialylated glycomoieties were used for performing affinity chromatography to separate serotonin [63]. Sialic acid is present at the end of the acidic glycan chains and known to have high affinity for serotonin as well as the sialic acid containing glycolipids and glycoproteins [64]. In 1982, Sturgeon et al. [65]

suggested that the presence of acetyl group and the side chain formed from C-7, C-8 and C-9 are necessary for binding of serotonin. Selective sides of sialic acid and serotonin to each other are marked in Figure 1.4.

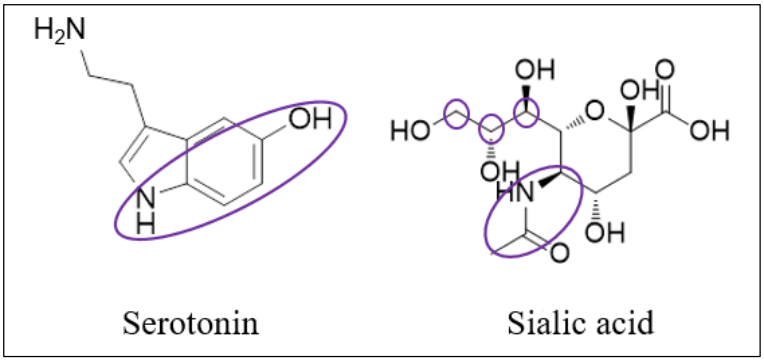


Figure 1.4. Selective sides of serotonin and sialic acid to each other

Availability of these binding sides while using sialic acid as a probe for biomolecules is crucial. Preparation of chromogenic probes like AuNPs by sialic acid results in oxidation of sialic acid which makes the usage of selective sides challenging. Lee et al. [30] reported that all five hydroxyl groups and N-acetyl group of sialic acid have the ability of reducing gold salt. In general, oxidation of sialic acid by sodium periodate and the products of this oxidation reaction were studied [35, 66]. The possible oxidation products depending on the amount of periodate and reaction conditions are given in Figure 1.5.

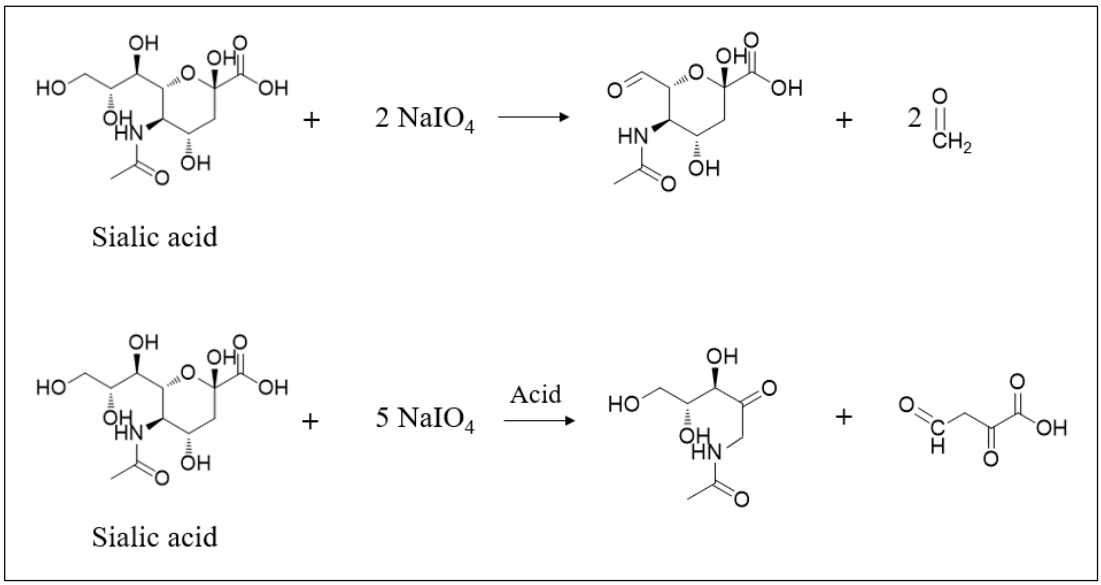


Figure 1.5. Possible oxidation products of sialic acid [35, 66]

1.5. Aim of Study

The aim of this thesis was to develop a simple, selective and sensitive method for serotonin determination. As a starting point, to use the affinity of serotonin for sialic acid, sialic acid stabilized AuNPs (SA-AuNPs) were synthesized. By using plasmonic properties of SA-AuNPs, serotonin determination was performed only by using a UV-VIS spectrophotometer. The basic principal was that the aggregation of SA-AuNPs increased with higher concentrations of serotonin and the absorption spectrum was red shifted accordingly. Once the affinity of SA-AuNPs for serotonin was proved, modifications of the AuNPs were done to enhance the aggregation and thus, to study the lower concentrations of serotonin. As a future study, applications of modified AuNPs on blood samples will be performed to determine serotonin levels in blood.

CHAPTER 2

EXPERIMENTAL

2.1. Chemicals and Reagents

In the preparation of all aqueous solutions, 18.2 MΩ.cm deionized, ultrapure water supplied from ELGA, Purelab Option-Q lab water purification system was used.

2.1.1. Synthesis of Sialic Acid Stabilized Gold Nanoparticles

N-(-)-Acetylneuraminic acid (96%, abcr), gold(III) chloride trihydrate ($HAuCl_4.3H_2O$, ≥99.9% trace metals basis, SIGMA-ALDRICH), sodium hydroxide (NaOH, puriss., 98-100.5%, pellets, SIGMA-ALDRICH), potassium bromide (for IR spectroscopy, MERCK) were used.

2.1.2. Sialic Acid Determination on the Surface of Gold Nanoparticles by MBTH Method

Sodium hydroxide (NaOH, puriss., 98-100.5%, pellets, SIGMA-ALDRICH), 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH, 97%, ALDRICH), DL-dithiothreitol (DDT, ≥98% (HPLC), SIGMA-ALDRICH), ammonium iron(III) sulfate dodecahydrate ($NH_4Fe(SO_4)_2.12H_2O$, ReagentPlus®, ≥99%, SIGMA-ALDRICH), sulfamic acid (ReagentPlus®, ≥99%, SIGMA-ALDRICH), hydrochloric acid (HCl, ACS reagent, 37%, SIGMA-ALDRICH) were used.

2.1.3. Addition of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to Gold Nanoparticles

2.1.3.1. Addition of EDC to Sialic Acid Stabilized Gold Nanoparticles

N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC.HCl, for sequence analysis, Fluka) was used.

2.1.3.2. Addition of EDC/NHS to L-Cysteine to Prepare Modified Gold Nanoparticles

Gold(III) chloride trihydrate ($HAuCl_4.3H_2O$, ≥99.9% trace metals basis, SIGMA-ALDRICH), trisodium citrate dehydrate (SIGMA-ALDRICH), L-cysteine (97%, ALDRICH), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC.HCl, for sequence analysis, Fluka), N-hydroxysuccinimide (NHS, 98%, SIGMA-ALDRICH), potassium bromide (for IR spectroscopy, MERCK) were used.

2.1.4. Preparation of Serotonin Solutions

Serotonin hydrochloride (98%, Alfa Aesar) was used.

2.1.5. Selectivity Studies of Prepared Gold Nanoparticles

Dopamine hydrochloride (SIGMA), L-Cysteine (97%, ALDRICH), L-aspartic acid (reagent grade, ≥98% (HPLC), SIGMA-ALDRICH), L-glutamic acid (ReagentPlus®, ≥99% (HPLC), SIGMA-ALDRICH) were used.

2.1.6. Synthesis of Cysteamine Coated Gold Nanoparticles

Gold(III) chloride trihydrate ($HAuCl_4.3H_2O$, ≥99.9% trace metals basis, SIGMA-ALDRICH), cysteamine (~95%, SIGMA-ALDRICH), sodium borohydride ($NaBH_4$, Merck) were used.

2.1.6.1. Conjugation of Carboxyl Group of Sialic Acid with AuNPs-NH_3^+

N-(-)-Acetylneuraminic acid (96%, abcr), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC.HCl, for sequence analysis, Fluka), N-hydroxysuccinimide (NHS, 98%, SIGMA-ALDRICH), MES hydrate (SIGMA) were used.

2.1.6.2. Conjugation of Carboxyl Group of Sialic Acid with AuNPs-NH_3^+ at pH 4

Triethylamine (pure, AKS), N-(-)-acetylneuraminic acid (96%, abcr), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC.HCl, for sequence analysis, Fluka), N-hydroxysuccinimide (NHS, 98%, SIGMA-ALDRICH), MES hydrate (SIGMA) were used.

2.1.6.3. Conjugation of Carboxyl Group of Sialic Acid with AuNPs-NH_3^+ at pH 5.5

Sodium hydroxide (NaOH, puriss., 98-100.5%, pellets, SIGMA-ALDRICH), N-(-)-acetylneuraminic acid (96%, abcr), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC.HCl, for sequence analysis, Fluka), N-hydroxysuccinimide (NHS, 98%, SIGMA-ALDRICH), MES hydrate (SIGMA) were used.

2.2. Instrumentation

2.2.1. Centrifugation

For collecting synthesized gold nanoparticles, Sigma 2-16P Bench-top Centrifuge and NÜVE NF 200 Small Centrifuge were used.

2.2.2. Ultrasonic Bath Sonicator

Elma Elmasonic S 40 Ultrasonic Bath was used for keeping temperature constant in experiments.

2.2.3. Ultraviolet-Visible Spectrophotometry

Colorimetric determination of serotonin by using functionalized AuNPs was made by measuring absorbance between 450 and 700 nm. Quartz cuvette and T80+ UV-VIS Instrument (PG Instruments Ltd.) was used for measurements.

2.2.4. Infrared Spectrophotometry

For qualitative characterization of functionalized AuNPs, Bruker Alpha T FT-IR Spectrometer was used.

2.2.5. Scanning Electron Microscopy

QUANTA 400F Field Emission Scanning Electron Microscope located in METU Central Laboratory was used for characterization of functionalized AuNPs.

2.2.6. Zetasizer Measurements

Sizes of the synthesized and serotonin added SA-AuNPs were measured by Malvern Mastersizer 2000 located in METU Central Laboratory.

2.2.7. pH Meter

The PHM210 Standard pH Meter was used for pH measurements of solutions.

2.2.8. Nutating Mixer

VWR® Nutating Mixer was used to shake prepared SA+EDC/NHS in 0.1 M MES buffer solution.

2.3. Procedures

2.3.1. Synthesis of Sialic Acid Stabilized Gold Nanoparticles

Synthesis of sialic acid functionalized gold nanoparticles was achieved by using reducing ability of sialic acid. 100 mL of 1.0 mM sialic acid solution was prepared in deionized water. To sialic acid solution, 600 μL of 0.035 M $HAuCl_4$ and 500 μL of 1.0 M NaOH solutions were added respectively. Prepared mixture was heated at 80°C for 15 min under continuous stirring at 1200 rpm [30]. At the end of the heating process, the color of the solution was changed from pale yellow to red color as shown in Figure 2.1. indicating the formation of sialic acid stabilized gold nanoparticles (SA-AuNPs).

Figure 2.1. SA-AuNPs having red color

The solution was let cool to room temperature and washed twice by centrifuging at 9000 rpm for 20 min. After removing supernatant, SA-AuNPs were dispersed in 30 mL deionized water and stored in refrigerator at 4 °C for further use.

For characterization of the surface functional groups, KBr pellet was used in sample preparation for taking the FT-IR spectrum of SA-AuNPs.

2.3.2. Determination of Sialic Acid on the Surface of Gold Nanoparticles by MBTH Method

The 3-Methyl-2-benzothiazolinone hydrazine (MBTH) method originally developed for determination of carbohydrates [42] was modified for determination of sialic acid on the surface of gold nanoparticles. In order to obtain a calibration plot, a standard solution of 0.02 M sialic acid was prepared in deionized water. From this standard solution, 100 μL sialic acid solutions having 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 nmol sialic acid were prepared in glass tubes. To each tubes, 100 μL of 0.5 N NaOH solution and 100 μL of MBTH reagent prepared by mixing equal volumes of 3.0 mg/mL MBTH and 1.0 mg/mL of DL-dithiothreitol (DDT) were added, respectively. The sample solutions were heated for 15 min at 80°C in a water bath. After removing the solutions from heat, 200 μL of acidic Fe solution containing 0.5% (w/v) $FeNH_4(SO_4)_2.12H_2O$, 0.5% (w/v) sulfamic acid and 0.25 N HCl was added and let to cool to room temperature. After cooling down, solutions were diluted to 1.0 mL with

deionized water followed by the measurement of absorbance at 620 nm [43] to draw a calibration plot.

To determine sialic acid on the surface, an indirect method was applied since the gold nanoparticles solution had red color. For this reason, colorless 50 μL sample of reaction mixture before synthesis of SA-AuNP was taken and treated same as the standard sialic acid solutions. After synthesis, the resulting AuNPs solution was centrifuged at 9000 rpm for 20 min so that the supernatant solution involving free sialic acid can be collected. 50 μL sample of this colorless supernatant solution was also taken to treat same as standard solutions followed by the measurement of absorbance at 620 nm. By the difference between initial amount of sialic acid and the amount of sialic acid in the supernatant solution, the amount of sialic acid used for the synthesis of SA-AuNPs was calculated.

2.3.3. Addition of Serotonin Solutions to Sialic Acid Stabilized Gold Nanoparticles

For addition of serotonin solutions to SA-AuNPs, a stock serotonin solution having 1.0 mM concentration was prepared with deionized water. This stock solution was used for the determination of serotonin having concentration between 100 – 500 μM. For determination between 10 – 100 μM, a second stock solution having 0.2 mM concentration was prepared from the former stock solution. Into 1.0 mL of SA-AuNPs solution, x mL of stock serotonin solutions diluted with (1-x) mL of deionized water were added to have desired concentration of serotonin solutions. The mixtures of SA-AuNPs and serotonin solutions were incubated at different temperatures and time intervals to observe color development of solutions caused by aggregation of nanoparticles. The absorbance values of the resulting solutions were measured by a UV-VIS spectrophotometer between 500 and 700 nm.

2.3.4. Addition of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to Gold Nanoparticles

To enhance aggregation of AuNPs induced by serotonin, in addition to affinity of serotonin to sialic acid from the selective side of the serotonin, the primary amine group of the serotonin can also be used for binding with functionalized AuNPs. Amine-carboxyl coupling by EDC was one of the easiest and effective ways of binding these two functional groups [67]. For this reason, addition of EDC to different carboxyl group containing functionalized AuNPS was performed. To modify a carboxyl group on the surface of gold nanoparticle, different procedures were applied.

2.3.4.1. Addition of EDC to Sialic Acid Stabilized Gold Nanoparticles

Firstly, carboxyl group of the sialic acid on the surface of SA-AuNPs was used for coupling with the amine group of serotonin by EDC. Figure 2.2. shows the proposed mechanism for the coupling reaction.

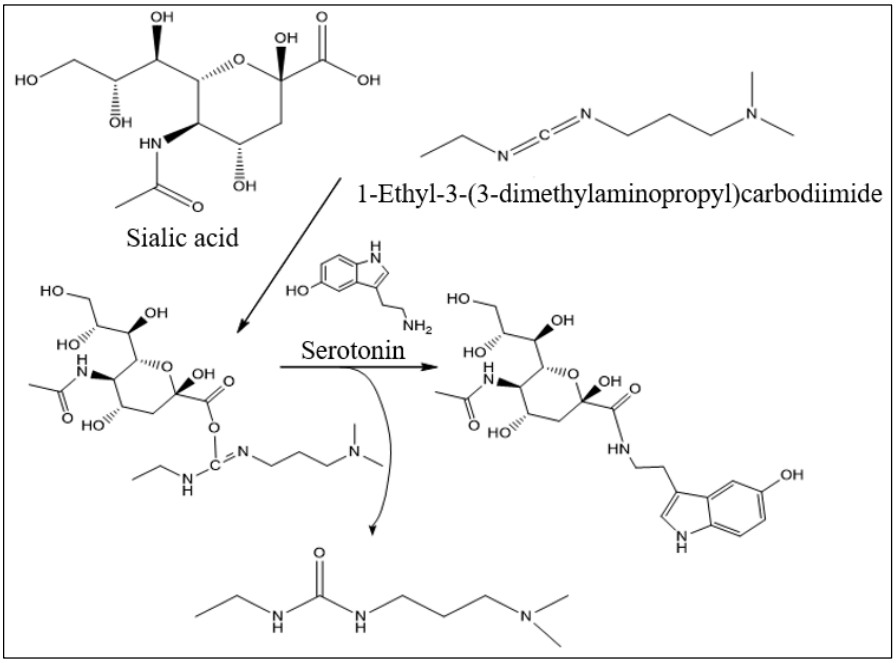


Figure 2.2. Reaction scheme of amine-carboxyl coupling between serotonin and sialic acid by EDC addition [67]

By MBTH method, it was calculated that 5.0 mL portion of SA-AuNPs contains 3.8 μmol sialic acid on the surface. The optimum amount of EDC added was calculated as two times mol of sialic acid on the surface. Addition of three fold EDC amount compared to sialic acid caused the change in the color of the solution. The color change from red to blue was due to the agglomeration of small gold nanoparticles. The amount of EDC added to 5.0 mL portion of SA-AuNPs was 380 μL of 0.02 M EDC solution which was prepared in deionized water. The prepared EDC solution was added to SA-AuNPs solution drop by drop, under continuous stirring at room temperature for 1 h. The resulting red solution was described as EDC+SA-AuNPs. The centrifuging of EDC+SA-AuNPs solution could not be performed due to agglomeration of particles.

To observe the effect of addition, two different experimental setups were prepared. The first set was prepared by mixing 0.5 mL of SA-AuNPs with 1.0 mL of serotonin solutions having concentration range between 84 and 500 μM for 15 min followed by the addition of 0.5 mL of EDC+SA-AuNPs solution. The second one was containing only 1.0 mL of EDC+SA-AuNPs mixed with same amount of serotonin solutions.

After deciding content of the solution, serotonin solutions having concentration ranges 0.05-5 μM and 10-100 μM were also added to study time and temperature of the reactions.

2.3.4.2. Addition of EDC/NHS to L-Cysteine to Prepare Modified Gold Nanoparticles

In order to replace ligands of the AuNPs with S containing L-cys molecules, citrate capped gold nanoparticles were synthesized by using Turkevich method [22]. 10.0 mL of 1.0 mM $HAuCl_4$ solution was heated to 90°C in a water bath. 5 min later, 1.0 mL of 38.8 mM trisodium citrate solution was added and heated at 90°C under stirring at 1200 rpm for 30 min. The resulting red solution was taken and cooled to room T followed by centrifuging at 10000 rpm for 25 min twice. The citrate capped AuNPs were dispersed in 10 mL deionized water and stored in refrigerator at 4°C for further use [68].

After synthesizing the citrate capped AuNPs, L-cysteine (L-cys) solution whose carboxyl group was activated by EDC/NHS addition were added to nanoparticles [69]. 1.0 mL of $2.0x10^{-4}$ M L-cys solution was prepared in deionized water and to this solution 1.0 mL of $2.0x10^{-3}$ M EDC and 1.0 mL of $2.0x10^{-3}$ M NHS solutions prepared in deionized water added, respectively [4]. The solution was stirred at 1200 rpm for 2 h at room temperature. To 5.0 mL portion of citrate capped AuNPs solution, 300 μL of prepared L-cys+EDC/NHS solution was added dropwise under stirring. This solution was also stirred at 1200 rpm for 2 h at room temperature. The centrifuging of resulting solution could not performed because of the agglomeration of particles.

Due to formation of Au-S covalent bond, the gold nanoparticles formed by attachment of the L-cys molecule were abbreviated as AuNPs-L-cys+EDC/NHS. After mixing equal volumes of SA-AuNPs with AuNPs-L-cys+EDC/NHS, serotonin solutions having 2.5, 6.7, 12.5 and 100 μM concentrations were added.

2.3.5. Selectivity Studies

To test the selectivity of particles for serotonin, dopamine, L-cysteine, L-aspartic acid and L-glutamic acid were used [49] since they were the only available ones in our laboratory.

2.3.5.1. Selectivity Study for Sialic Acid Stabilized Gold Nanoparticles

600 μM of dopamine, L-cysteine, L-aspartic acid and L-glutamic acid stock solutions were prepared in deionized water. 300 μM of these solutions were added to 1 mL portions of SA-AuNPs incubated at room temperature for 1 h. Absorbance values of the resulting solutions were measured by a UV-VIS spectrophotometer between 450 and 700 nm.

2.3.5.2. Selectivity Study for EDC Added Sialic Acid Stabilized Gold Nanoparticles

300 μM of dopamine, L-cysteine, L-aspartic acid and L-glutamic acid solutions prepared in deionized water were added to 1.0 mL of EDC+SA-AuNPs and the

solutions were incubated at room temperature for 30 min. Finally, absorbance values of the resulting solutions were measured by a UV-VIS spectrophotometer between 450 and 700 nm.

2.3.6. Synthesis of Cysteamine Coated Gold Nanoparticles

To attach sialic acid on the AuNPs surface only from its carboxyl group, amine-carboxyl coupling with EDC/NHS mechanism was aimed. For preparing AuNPs having amine group at the end of the ligands, synthesis of cysteamine coated AuNPs abbreviated as AuNPs-NH_3^+ was performed according to following procedure [70]. In to 40.0 mL of 1.42 mM $HAuCl_4$ solution, 400 µL of 213.0 mM cysteamine solution was added under stirring at 1200 rpm, at room temperature. After further stirring for 20 min, 10 µL of 10.0 mM freshly prepared $NaBH_4$ solution was added under vigorous stirring. The solution further stirred for 60 min in dark and stored at 4°C. The scheme of the experiment is shown in Figure 2.3.

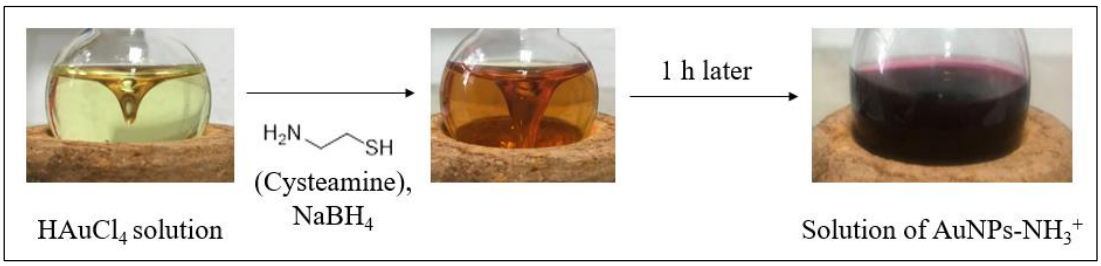


Figure 2.3. The reaction scheme of synthesis of AuNPs- NH_3^+

For characterization of the surface functional groups, KBr pellet was used in sample preparation for taking the FT-IR spectra of SA and SA+EDC/NHS added AuNPs-NH_3^+.

2.3.6.1. Conjugation of Carboxyl Group of Sialic Acid with AuNPs-NH_3^+

For conjugating the sialic acid with amine group of AuNPs-NH_3^+, firstly, carboxyl group of sialic acid was activated by addition of EDC/NHS. Firstly, 0.1 M pH 5 MES buffer was prepared since pH 5 is optimum condition for this conjugation [4]. 5.9 mg of sialic acid was dissolved in MES buffer followed by the dissolution of 1.91 mg of EDC and 2.88 mg of NHS and mixed at room T for 15 min by shaker. This solution was transferred to 9.0 mL of AuNPs-NH_3^+ solution and incubated at 50°C under stirring at 750 rpm for 3 h [71]. The prepared solution was dialyzed against deionized water for 1 h. Into 1.0 mL portions of dialyzed solution, serotonin solutions having concentrations between 100 and 500 μM were added to observe if there was any aggregation caused by serotonin. The proposed reaction between cysteamine and sialic acid whose carboxyl group was activated by addition of EDC/NHS is shown in Figure 2.4.

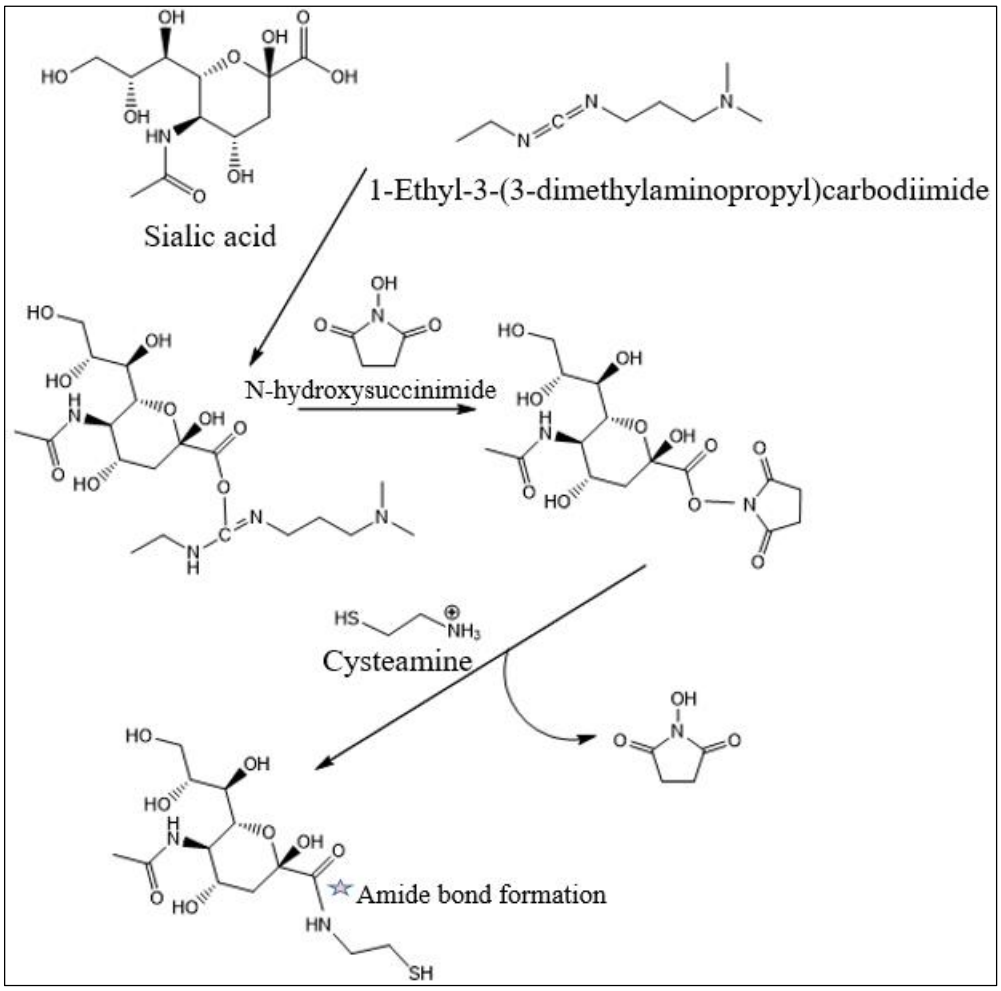


Figure 2.4. The proposed reaction between cysteamine and sialic acid [67]

2.3.6.2. Conjugation of Carboxyl Group of Sialic Acid with AuNPs-NH_3^+ at pH 4

Addition of an organic base, triethylamine (TEA), was performed to bring the pH of AuNPs-NH_3^+ solution close to buffer solution used for EDC/NHS addition to sialic acid. Hence, 5.0 mL portion of AuNPs-NH_3^+ solution was taken to add 1.5 μL of TEA, in 1:1 mol ratio with cysteamine on the surface, and mixed for 2.5 h. On the other hand, 3.3 mg of sialic acid, 1.0 mg of EDC and 1.2 mg of NHS dissolved in 556 μL of 0.1 M pH 5 MES buffer respectively. Prepared buffer solution was added to AuNPs-NH_3^+ solution after 15 min mixing with a shaker and this solution was incubated at 50 °C under stirring at 750 rpm for 3 h [71]. The resulting solution was dialyzed against deionized water for 1 h. 1.0 mL portions of dialyzed solution were taken to add serotonin solutions having concentrations between 100 and 500 μM to observe if there was any aggregation induced by serotonin.

2.3.6.3. Conjugation of Carboxyl Group of Sialic Acid with AuNPs-NH_3^+ at pH 5.5

After considering the unwanted reaction of TEA with EDC/NHS since it is an amine containing base, NaOH was decided to use for bringing the pH of AuNPs-NH_3^+ solution same as buffer solution that sialic acid was prepared. Into 5.0 mL portion of AuNPs-NH_3^+ solution, 400 μL of 0.01 M NaOH solution was added dropwise under stirring. Once pH of the AuNPs-NH_3^+ solution was increased to 5.5, the solution of 3.3 mg of sialic acid, 1.0 mg of EDC and 1.2 mg of NHS dissolved in 556 μL of 0.1 M pH 5 MES buffer was added after 15 min of mixing. The resulting solution was dialyzed against deionized water for 1 h. 1.0 mL portions of dialyzed solution were taken to add serotonin solutions having concentrations between 100 and 500 μM to observe if there was any aggregation induced by serotonin.

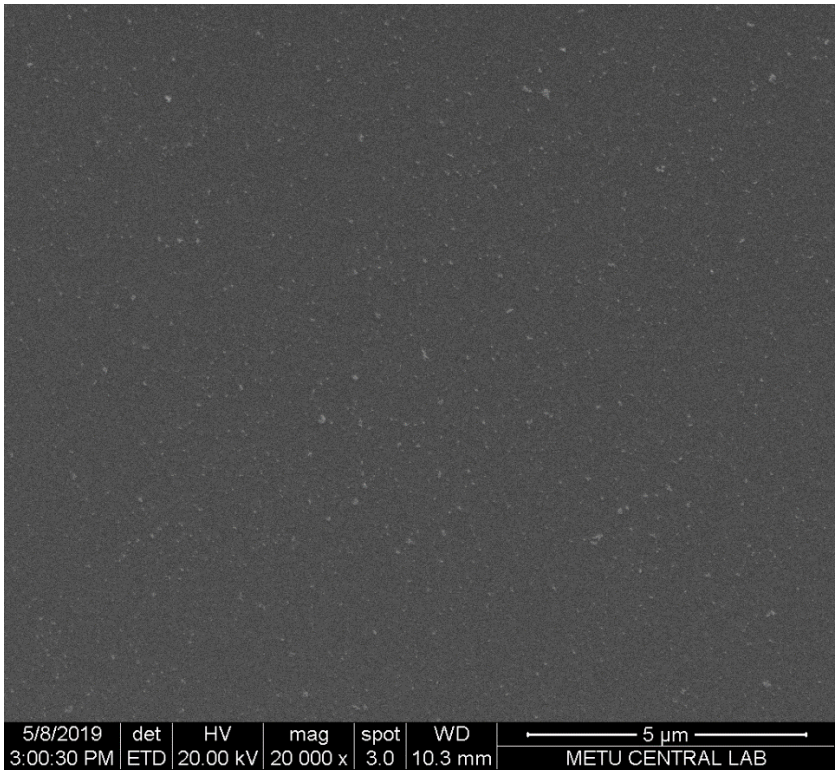
CHAPTER 3

RESULTS AND DISCUSSION

In the scope of this thesis, serotonin determination based on affinity of serotonin to sialic acid [65] was aimed by using a simple colorimetric method. In addition to synthesis of sialic acid stabilized gold nanoparticles, to enhance aggregation of gold nanoparticles upon addition of serotonin, different modifications of the gold nanoparticles were done. This modification of particles was monitored by using UV-VIS Spectrophotometry and FT-IR spectrophotometry. In addition, aggregation of AuNPs was followed by the SEM images. Size measurements of these nanoparticles were done by Zetasizer.

3.1. Preparation of Sialic Acid Stabilized Gold Nanoparticles

When synthesizing sialic acid functionalized gold nanoparticles, using reducing ability of sialic acid was enough to obtain gold nanoparticles having red wine color [30]. Figure 3.1. shows the scanning electron microscope (SEM) images of SA-AuNPs. From the figure, monodispersity and the spherical shape of gold nanoparticles due to sialic acid on the surface can be seen.



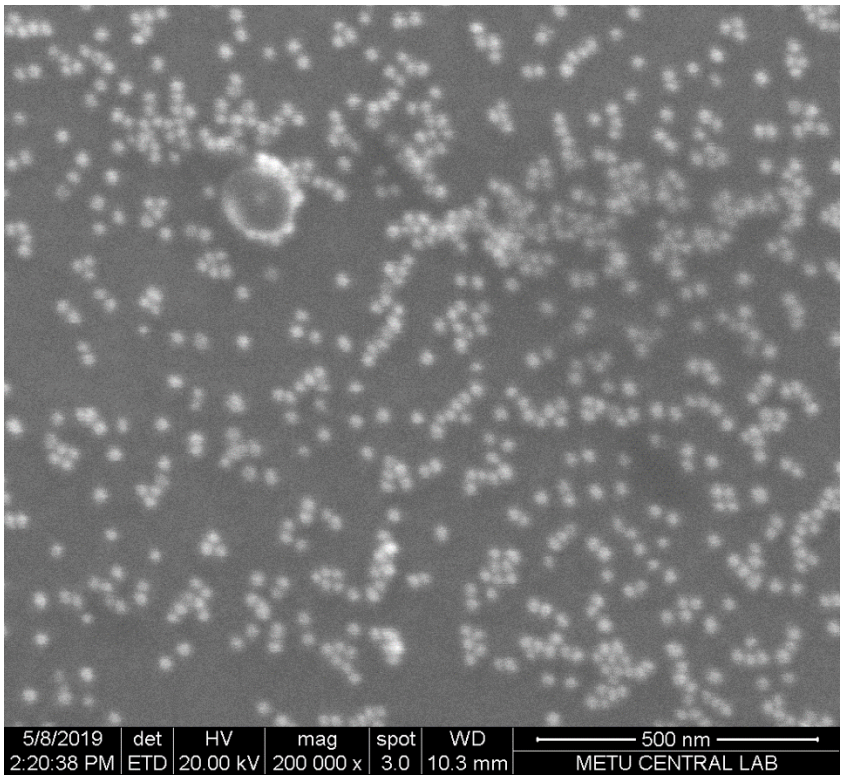


Figure 3.1. SEM images of SA-AuNPs

The presence of gold nanoparticles was proved by the energy-dispersive X-ray (EDX) spectroscopy. EDX pattern of the SA-AuNPs can be seen in Figure 3.2.

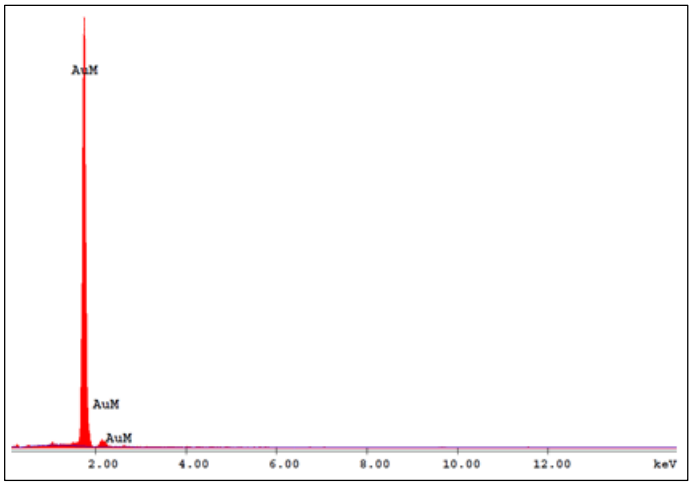


Figure 3.2. EDX pattern of SA-AuNPs

In addition to these analyses, size measurement of the synthesized SA-AuNPs was performed by a zetasizer instrument and the resulting size distribution graph is given in Figure 3.3. The average size of the SA-AuNPs was calculated as 21 ± 3 nm by ImageJ programme.

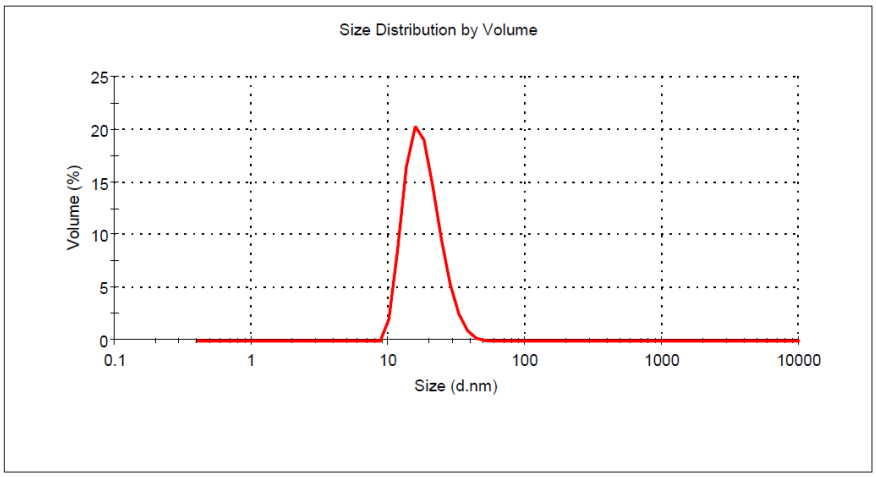


Figure 3.3. Size distribution graph of SA-AuNPs

For examining the presence of sialic acid on the surface of gold nanoparticles, FT-IR spectrum of SA-AuNPs was taken. From the spectrum shown in Figure 3.4., the peaks at 1646, 1514, 1455 and 1316 cm^{-1} indicate the C=O stretching, N-H bending, C-N stretching and C-O stretching [72] relatively and prove the presence of sialic acid on the surface.

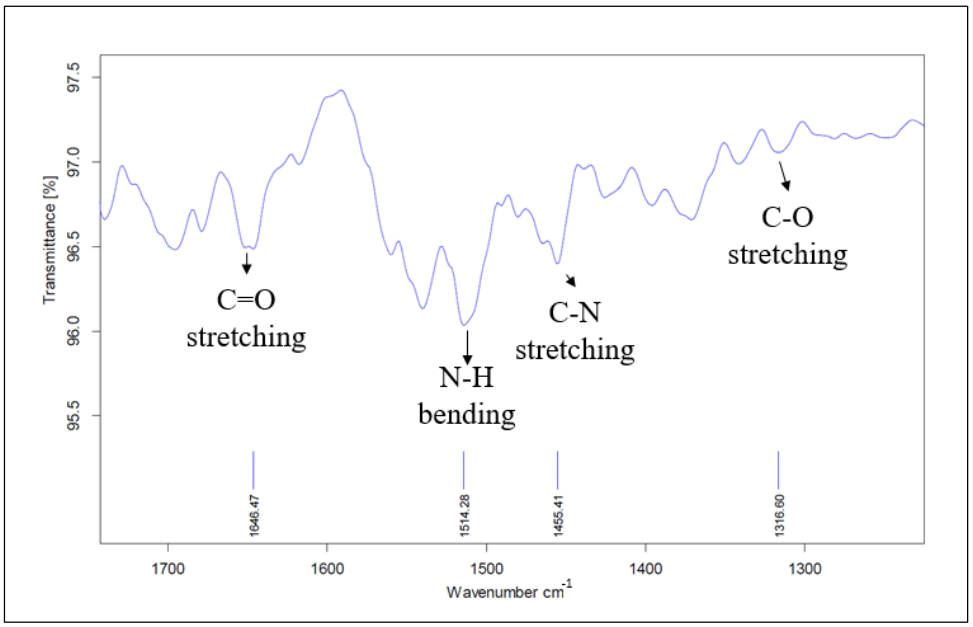


Figure 3.4. FT-IR spectrum of SA-AuNPs

3.2. Determination of Sialic Acid on the Surface of Gold Nanoparticles by MBTH Method

It is important to synthesize small gold nanoparticles if these nanoparticles are going to be used in developing a colorimetric method as the nanoparticles get bigger, a red shift in the spectrum occurs [73]. Therefore, it was essential to determine the optimum amount of sialic acid for the synthesis of smaller SA-AuNPs.

According to Lee at al., 1.25 mM sialic acid concentration is needed to synthesize particles having diameter of approximately 20 nm [1]. However, when the described procedure was applied, purple solution of gold nanoparticles indicating larger AuNPs [73] was obtained. Hence, a study on the amount of reagents added for synthesis was necessary. To determine the amount of sialic acid needed for smaller gold nanoparticles, the 3-methyl-2-benzothiazolinone hydrazine (MBTH) method was used. The usage of MBTH method on the determination of sialic acid without any former oxidation step was reported for the first time in this study. The resulting

product of the reactions was a blue colored cationic dye which had an absorbance at 620 nm. Figure 3.5. shows the reaction scheme.

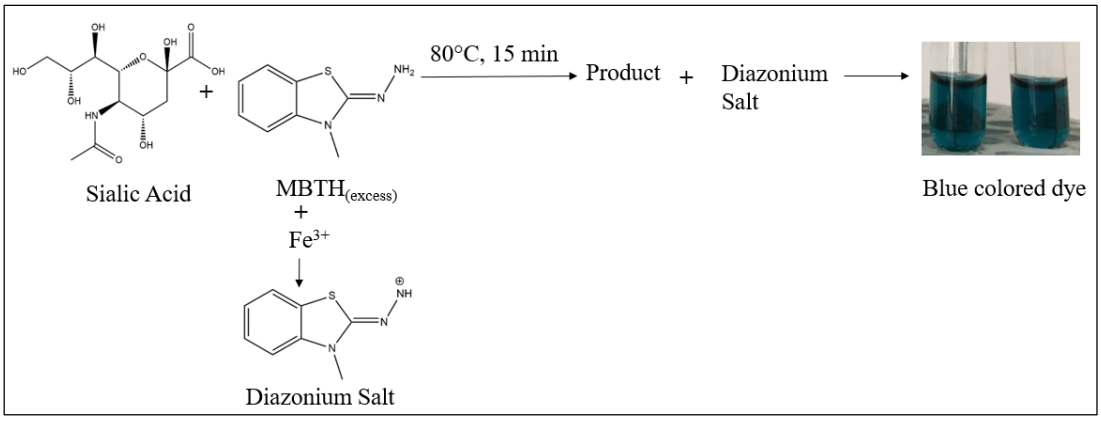


Figure 3.5. Reaction scheme of MBTH method applied on sialic acid

The intensity of the absorbance signal at 620 nm increased when the concentration of sialic acid increased providing a calibration plot of standard sialic acid solutions as shown in Figure 3.6.

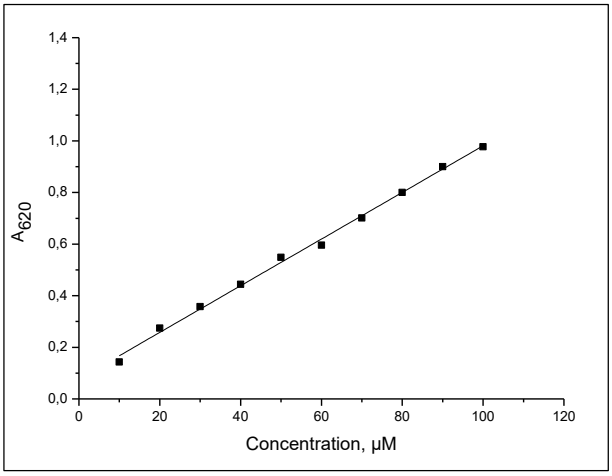


Figure 3.6. Calibration plot of standard sialic acid solutions having concentration of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM

Table 3.1. *Line equation, R^2, LOD and LOQ values derived from calibration plot*

Line Equation	**R^2**	**LOD (μM)**	**LOQ (μM)**
y=9040x+0.077	0.9969	0.35	1.17

Line equation, R-squared (R^2), limit of detection (LOD) and limit of quantitation (LOQ) values derived from the calibration plot were given in Table 3.1. LOD and LOQ values were calculated by measuring the absorbance of blank solution ten times and then the standard deviation of absorbance values of this blank solution was multiplied by 3 for LOD and 10 for LOQ and divided by slope. As it can be concluded from the table, the described method can be successfully used for determination of sialic acid in μM levels.

To determine the amount of sialic acid used for the synthesis of gold nanoparticles, an indirect measurement was used since SA-AuNPs solution had a red wine color disabling the absorbance measurement of blue colored product of MBTH method. For this reason, colorless 50 μL sample of reaction mixture before synthesis of SA-AuNP and 50 μL sample of colorless supernatant solution involving free sialic acid after centrifuging the synthesized SA-AuNPs were taken for measurement of sialic acid with MBTH method. After applying MBTH method, the amount of sialic acid used for the gold nanoparticles synthesis was calculated by the difference between the overall amount of sialic acid and the free sialic acid remaining in the supernatant. Various concentrations of sialic acid solutions were added to reaction mixture for obtaining the red color of gold nanoparticles with highest amount of sialic acid used for the synthesis. The amount of gold salt was kept constant during these studies. All concentrations studied were in the working range since LOD and LOQ values for MBTH method were much lower than the concentration range that was studied.

Table 3.2. *Amount of sialic acid used for the synthesis of SA-AuNPs and the color of the solutions*

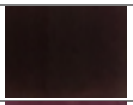
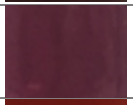
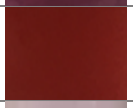
Overall Amount of Sialic Acid (μmol)	**Amount of Sialic Acid in the Supernatant (μmol)**	**Amount of Sialic Acid Used for the Synthesis (μmol)**	**Color of the Solutions**
27.20	26.22	0.98	
12.70	11.71	0.99	
10.30	8.70	1.60	
4.88	4.44	0.44	

Table 3.2. shows that highest amount of sialic acid used for the synthesis was obtained when 10.30 μmol sialic acid was added in the reaction mixture. In the presence of higher amount of sialic acid, the resulting AuNPs solutions had dark purple color indicating agglomeration of nanoparticles. The reason for that might be the hydrogen bonding between free sialic acid with the sialic acid on the surface of nanoparticles. On the other hand, when the amount of sialic acid was low, AuNPs solution had a pale purple color. The decrease in charge repulsion between nanoparticles due to low amount of sialic acid might cause of agglomeration of nanoparticles resulting in a pale purple color. As a result, initial sialic acid concentration was chosen as 1.0 mM and the optimum mol ratio between Au^{3+} and sialic acid was calculated as 0.21:1.00.

3.3. Determination of Serotonin by Using Sialic Acid Stabilized Gold Nanoparticles

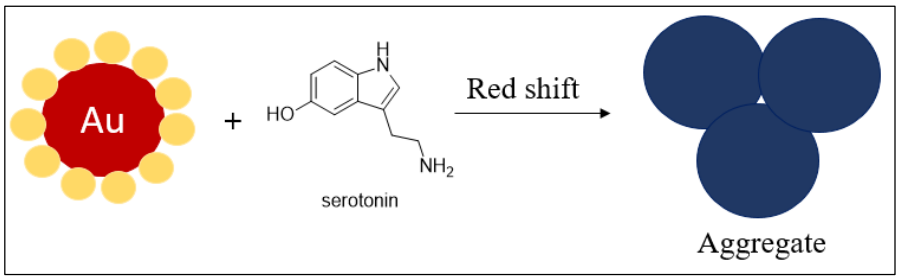
Different concentration ranges of serotonin solutions were studied for the determination of serotonin.

The first concentration range was chosen between 80 and 500 μM. The aggregation rate of the SA-AuNPs depending on the concentration of serotonin solutions was

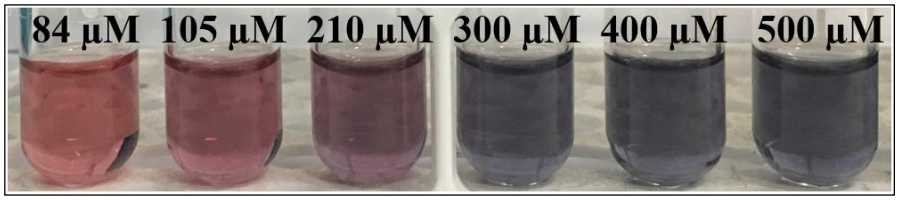
differed at different temperatures. A red shift in the spectrum was expected as nanoparticles got bigger due to interaction between sialic acid and serotonin [65]. Therefore, the ratio of absorbance at 650 nm to that at 520 nm was taken as ordinate of calibration plot in following studies. The addition of serotonin solution for each concentrations was done at 10 min intervals to be able to measure absorbance of each solution exactly after the specified time since aggregation increases with time.

Figure 3.7. shows the reaction scheme of serotonin with SA-AuNPs, color scheme of solutions, red shift in the spectrum and calibration plot drawn by taking the ratio of absorbance at 650 nm and 520 nm for 80 °C temperature since that ratio gave the best slopes.

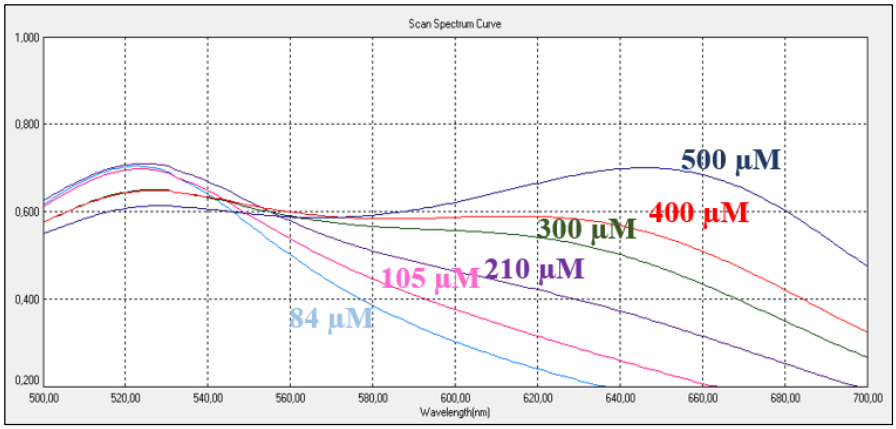
(a)



(b)



(c)



(d)

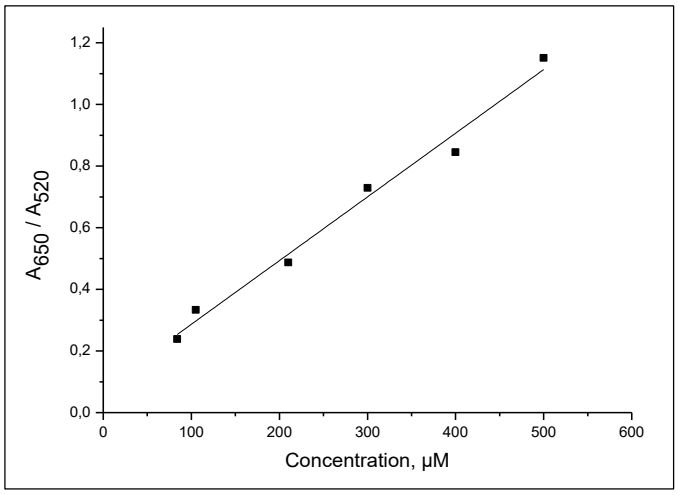


Figure 3.7. (a) Reaction scheme, (b) Color scheme of solutions, (c) Red shift in the absorbance spectrum and (d) Calibration plot obtained 10 min after addition of 84, 105, 210, 300, 400, 500 µM serotonin solutions respectively at 80 °C

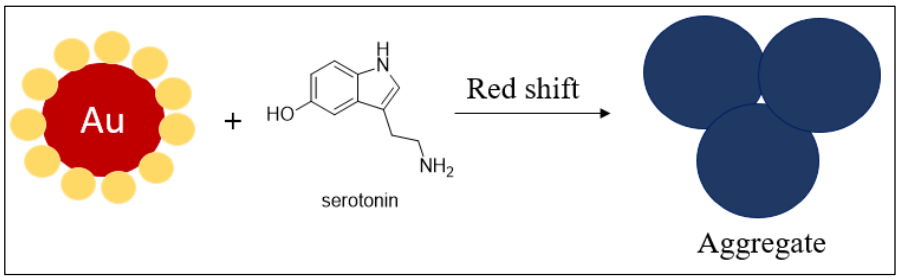
Table 3.3. *Reaction time, line equation, R^2, LOD and LOQ at different temperatures*

Temperature (°C)	Reaction Time (min)	Line Equation	R^2	LOD (μM)	LOQ (μM)
25	60	y=0.0021x-0.0268	0.9782	2.71	9.04
80	10	y=0.0021x+0.0800	0.9858	1.09	3.63

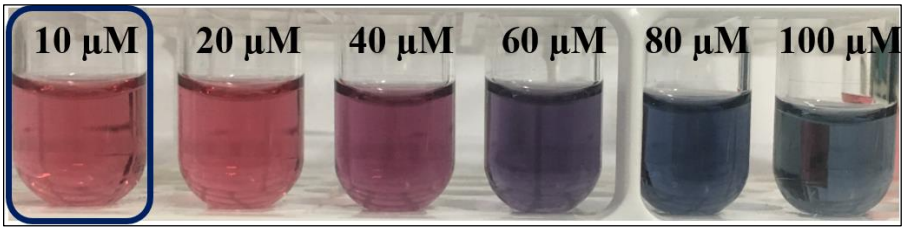
Table 3.3. clearly shows that increasing the temperature caused a significant decrease in reaction time whereas the slope of the curve did not change. Since the measurements were time depended, the absorbance of solutions was measured only once. However, the slope for this concentration range was found as 0.0021 ± 0.0002 where s is the propagation of error from four sets of measurements done in different days showing the consistency of the measurements. LOD and LOQ values were calculated as 1.09 μM and 3.63 μM for 80 °C. The LOD was relatively high when comparing other colorimetric method [49] which was 0.1 μM. However, in practice, it was not possible to obtain a color development for concentrations lower than 80 μM within the time interval and temperature given. Therefore, the calculation of LOD and LOQ values was not performed in further experiments since the minimum concentration of serotonin causing aggregation was already in the calibration curve.

The second concentration range of serotonin was between 10 and 100 μM. As it can be concluded from the first concentration range, working at room temperature would not be a practical choice for lower concentrations in terms of reaction time. Consequently, 80°C was chosen to study in this concentration range. After 60 min at 80°C water bath, color development of the solutions was completed except the one having 10 μM serotonin. Since 10 μM serotonin concentration was not enough to cause aggregation, the calibration curve was plotted by rejecting the absorbance data coming from that solution. The reaction scheme of serotonin with SA-AuNPs , color scheme of the solutions, red shift in the absorbance spectrum and calibration plot drawn by taking the ratio of absorbance at 620 nm and 520 nm are shown in Figure 3.8.

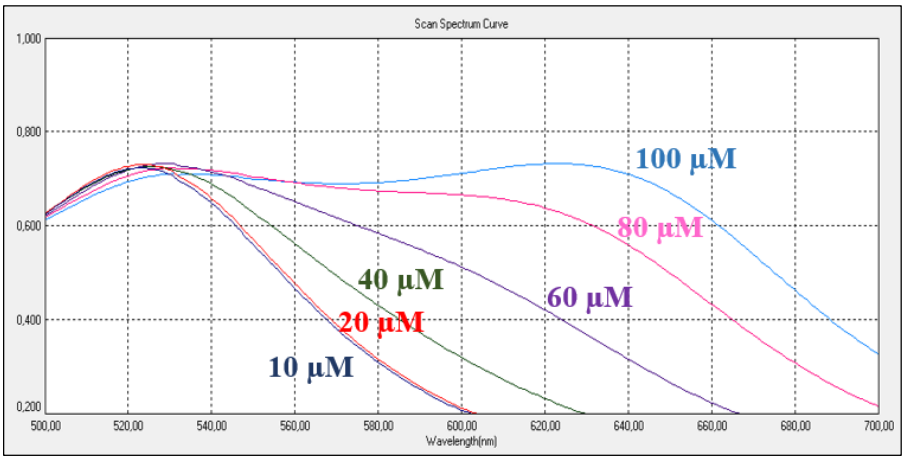
(a)



(b)



(c)



(d)

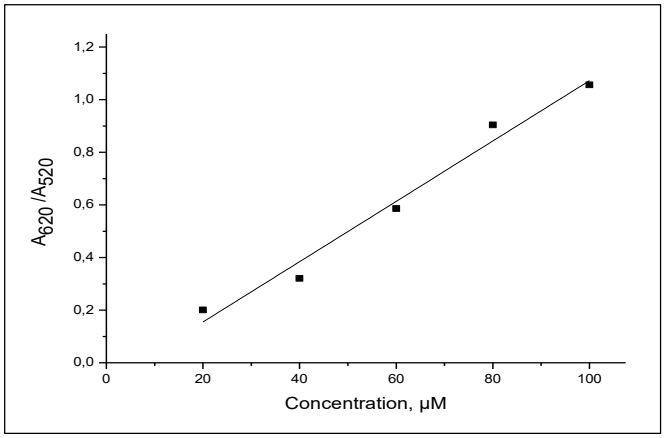


Figure 3.8. (a) Reaction scheme, (b) Color scheme of solutions, (c) Red shift in the absorbance spectrum and (d) Calibration plot obtained 60 min after addition of 20, 40, 60, 80, 100 µM serotonin solutions respectively at 80 °C

From the calibration plot line equation was obtained as y=0.0115x-0.0743 and R^2 was 0.9797. The slope for this concentration range was found as 0.0113 ± 0.0020 where s is the propagation of error from four sets of measurements. The ratio of absorbance at 620 nm to that at 520 nm was taken for calibration plot because it gave higher slopes with highest R^2. LOD and LOQ values were not calculated because as explained in page 37, there was no color change resulting from the addition of serotonin concentration lower than 10 μM for the time interval and temperature given. As a result, the concentration range of serotonin solutions to draw calibration plot in Figure 3.8. was the limit that can be measured by this method.

When comparing the two solutions having 84 μM and 80 μM serotonin concentration, it can be clearly seen that the increase in reaction time causes red shift in the spectrum resulting from the aggregation of nanoparticles shown in Figure 3.9. That is the reason of having different absorbance ratios for similar concentration of serotonin measured at different time. Therefore, optimization of reaction time for lower concentrations was another important way of enhancing aggregation in addition to reaction temperature.

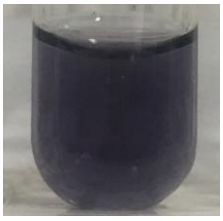
(a)  (b) 

Figure 3.9. (a) 10 min after addition of 84 μM serotonin and (b) 60 min after addition of 80 μM serotonin

To have aggregation of nanoparticles caused by 10 μM serotonin solution, the solution was heated up to 92°C for 4 hours and let to stand in dark for several days but no change in the color of the solution was observed. On the contrary, color of the one containing 20 μM serotonin turned to blue next day and a day later, the nanoparticles

precipitated remaining a colorless supernatant at the top. As a result, serotonin concentrations lower than 20 μM cannot be studied by just using SA-AuNPs. Different modifications of AuNPs made to be able to work with lower concentration range of serotonin will be explained in the following studies.

3.4. Addition of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to Gold Nanoparticles

In addition to selective side of serotonin for aggregation of SA-AuNPs, the primary amine group of it was also available for binding with functionalized AuNPs. For achieving amine-carboxyl coupling, EDC was added to functionalized AuNPs.

3.4.1. Addition of EDC to Sialic Acid Stabilized Gold Nanoparticles

Two different particle mixtures were prepared to study on effect of EDC addition. After addition of EDC to SA-AuNPs, centrifuging of resulting solution could not be performed due to agglomeration of particles. In the presence of EDC, the formed black aggregates could not be dispersed again. Therefore, excess EDC could not be discarded from AuNPs solutions. Nevertheless, it was hoped that excess EDC did not react with serotonin and cause interference. In addition, the black precipitate of AuNPs was also observed 1 day after adding serotonin solutions with high concentration to only SA-AuNPs and once the color turned black with precipitation, dispersion of nanoparticles was not possible for those nanoparticles too.

For the first particle mixture, the color development was achieved 15 min after addition of EDC+SA-AuNP to SA-AuNPs+Serotonin (84-500 μM) solutions. Before adding EDC+SA-AuNPs, serotonin solutions having 84, 105, 210, 300, 400, 500 μM concentration were added to SA-AuNPs solutions and let to react for 15 min. Thus, total reaction time was 30 min. Figure 3.10. shows the expected reaction scheme of serotonin with the SA-AuNPs and EDC+SA-AuNPs.

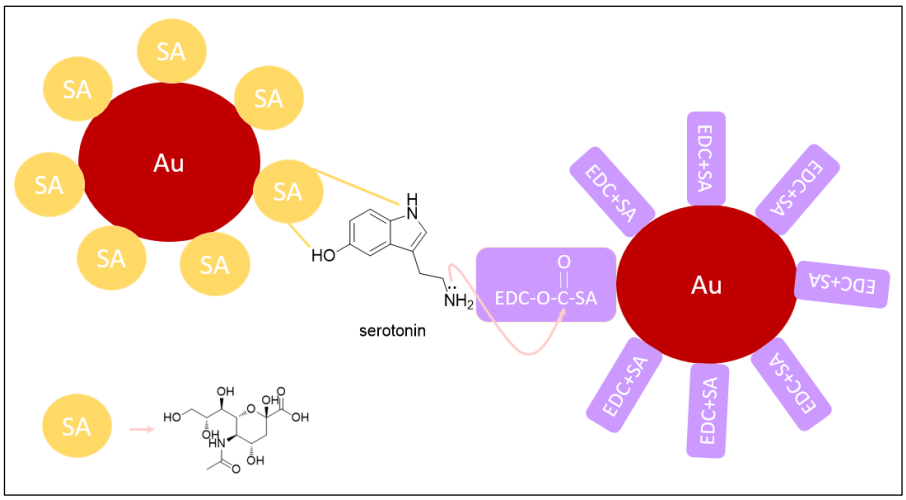


Figure 3.10. The reaction scheme of serotonin with SA-AuNPs and EDC+SA-AuNPs

Table 3.4. *The reaction time and line equation, R^2 for serotonin determination with the particle mixture of SA-AuNPs and EDC+SA-AuNPs at 25°C*

Reaction Mixture	**Reaction Time (min)**	**Line Equation**	**R^2**
SA-AuNPs + Serotonin (84, 105, 210, 300, 400, 500 µM) + EDC+SA-AuNPs	30	y=0.0024x-0.113	0.9359

From the Table 3.4., it can be seen that the total reaction time was 30 min. As shown in the reaction scheme, Figure 3.10., in the presence of excess EDC, it was expected that all the sialic acids (SA) were modified with EDC, hence no agglomeration was expected when these particles were used alone for serotonin determination. In order to evaluate our argument same concentrations of serotonin solutions were added to

aqueous solution of EDC+SA-AuNPs. Figure 3.11. is showing the estimated reaction scheme for the reaction of serotonin with the EDC+SA-AuNPs.

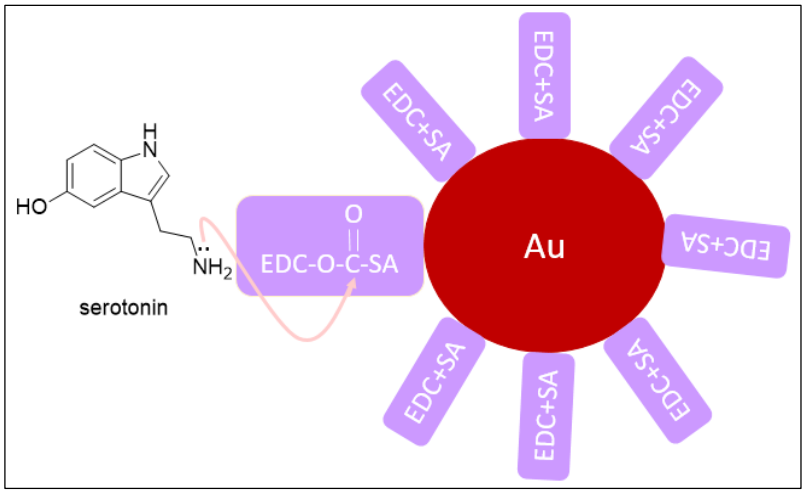


Figure 3.11. The reaction scheme of serotonin with EDC+SA-AuNPs

Table 3.5. *The reaction time and line equation, R^2 for serotonin determination with EDC+SA-AuNPs at 25°C*

Reaction Mixture	**Reaction Time (min)**	**Line Equation**	**R^2**
Only EDC+SA-AuNPs + Serotonin (84, 105, 210, 300, 400, 500 µM)	30	y=0.0025x-0.0253	0.9941

The line equation and R^2 derived from the calibration plot is given in Table 3.5. The slope for this concentration range was found as 0.0025 ± 0.0024 where s is the propagation of error from four sets of measurements. The enhanced aggregation by

using only EDC+SA-AuNPs solution showed that not all the EDC molecules were attached to all sialic acid molecules on the surface so that aggregation by using two sides of the serotonin molecules was achieved. Therefore, the reaction scheme in Figure 3.11. should be corrected as given in Figure 3.12.

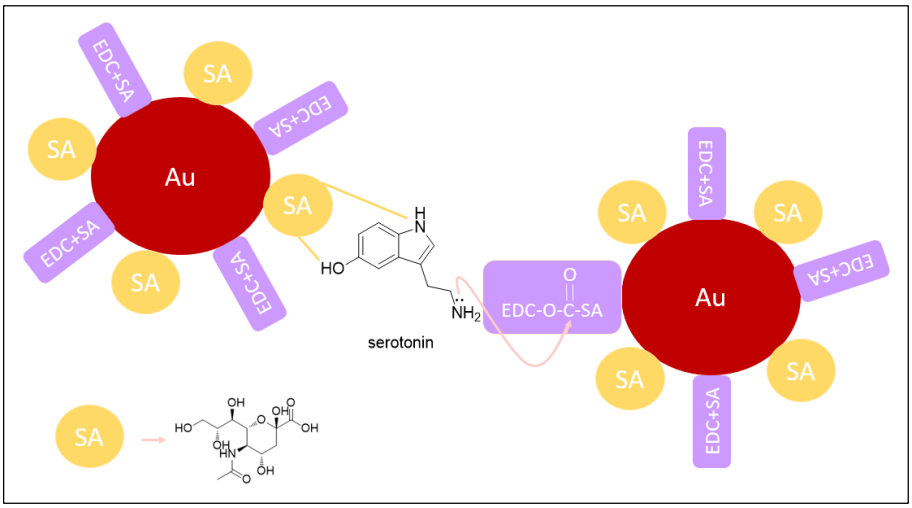


Figure 3.12. The reaction scheme of serotonin with EDC+SA-AuNPs

Once the particle mixture was decided, studies on different concentrations of serotonin were started.

Table 3.6. *The reaction time and aggregation of EDC+SA-AuNPs for different serotonin concentration ranges at 25°C*

Concentrations of serotonin solutions (µM)	**Reaction Time at Room T**	**Aggregation**
0.05 - 5	1 h	✗
0.05 - 5	2.5 h	✗
5 - 100	1 h	✓
80-500	30 min	✓

Table 3.6. shows that at room T, no aggregation of EDC+SA-AuNPs was observed for serotonin concentrations lower than 5 μM even after 2.5 h. Hence, optimization study for temperature to work with lower concentrations was performed.

For the concentration range between 80 and 500 μM, 30 min at room temperature was enough to aggregate nanoparticles as mentioned. Figure 3.13. shows the color scale of gold nanoparticles solutions 30 min after addition of serotonin solutions in that concentration range.

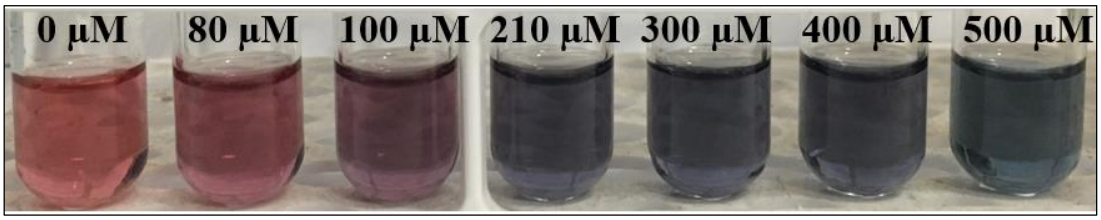


Figure 3.13. Color scale of gold nanoparticles solutions 30 min after addition of serotonin solutions

In order to monitor aggregation of nanoparticles resulting from the addition of serotonin, scanning electron microscope (SEM) images of three different solutions were taken. Figure 3.14. shows SEM images of a blank solution of EDC+SA-AuNPs and the aggregation of EDC+SA-AuNPs 30 min after addition of 100 and 500 μM serotonin respectively. From the images, it can be concluded that when the concentration of serotonin increased, the small EDC+SA-AuNPs were getting larger due to aggregation and the color of solution was changing from red to blue.

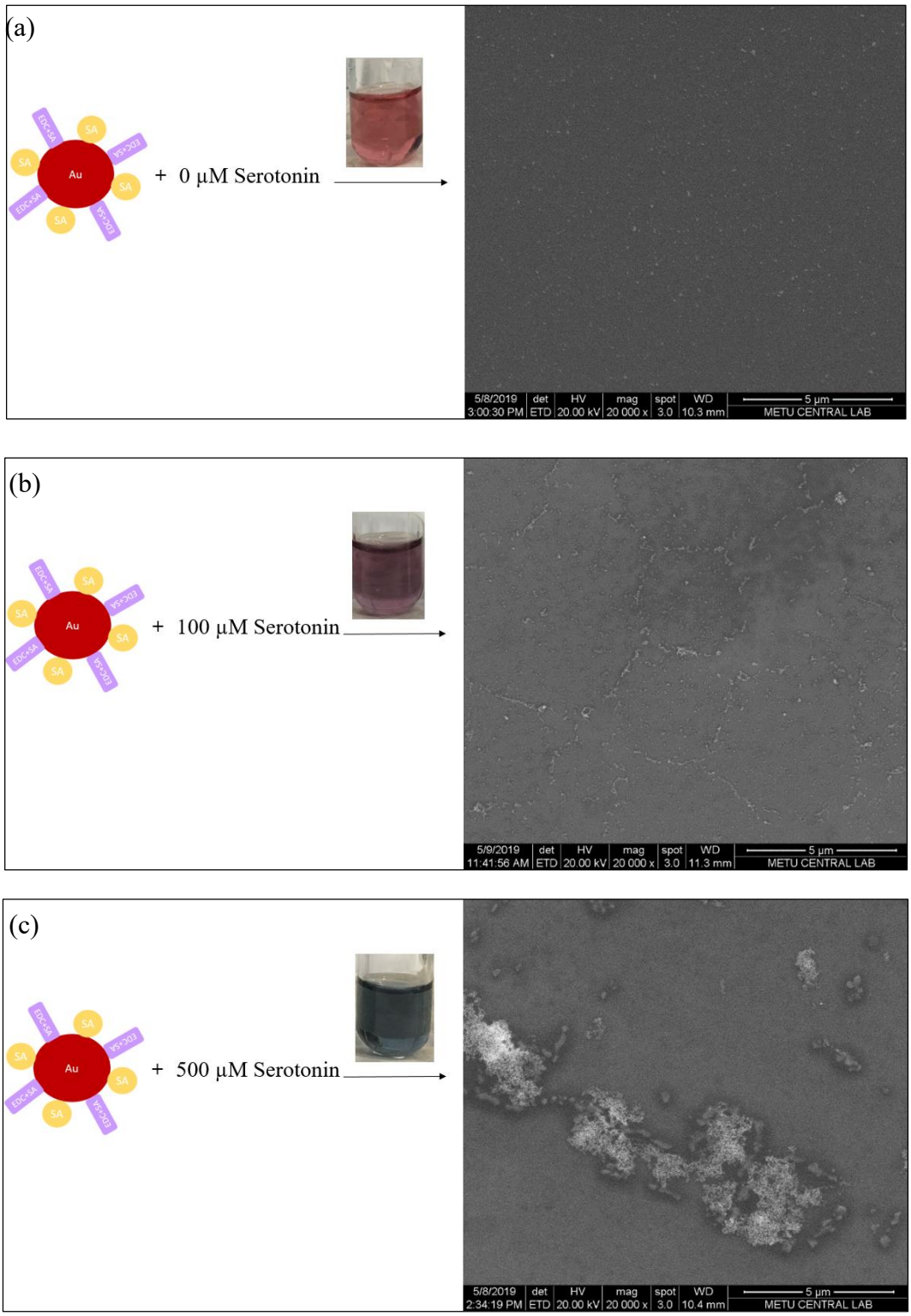


Figure 3.14. SEM images of EDC+SA-AuNPs 30 min after addition of (a) 0 µM, (b) 100 µM and (c) 500 µM serotonin

For concentration between 5 and 100 µM, the reaction time was increased to 1 h at room temperature. Since 1 h is not a long reaction time and studying at room temperature is better in terms of ease of the experiment, no optimization of temperature was done for this concentration range. The obtained calibration plot from this study is shown in Figure 3.15.

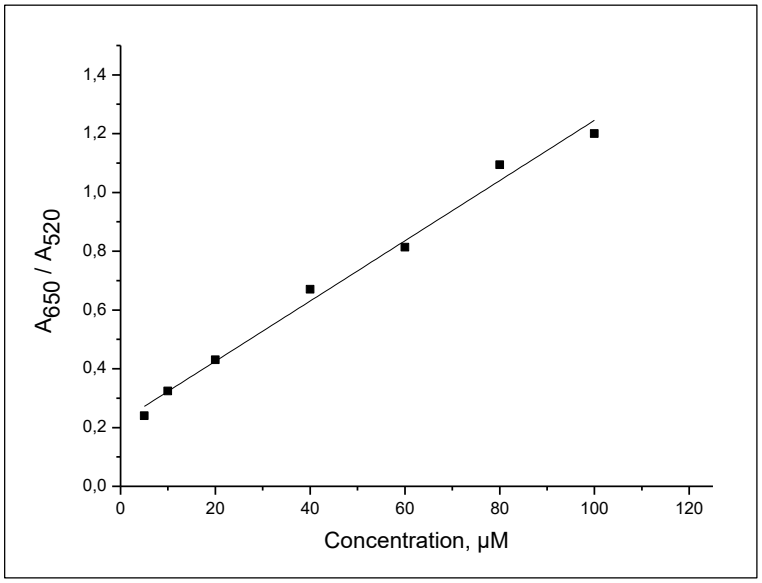


Figure 3.15. Calibration plot after addition of 5, 10, 20, 40, 60,80 and 100 µM serotonin solutions to EDC+SA-AuNPs at 25°C

From the calibration plot, line equation was obtained as y=0.0102x+0.2206 and R^2 was 0.9905. The slope for this concentration range was found as 0.0103 ± 0.0016 where s is the propagation of error from four sets of measurements. From the results of the experiment, the lowest concentration that we can determine with EDC+SA-AuNPs was 5 µM.

When working with concentration range between 0.05 and 5 µM, however, despite of increasing temperature up to 80°C and reaction time to 1 day, no aggregation upon addition of serotonin was observed.

Table 3.7. *The reaction time and aggregation of EDC+SA-AuNPs at different temperatures upon addition of 0.05-5 μM serotonin*

Temperature (°C)	Reaction Time	Aggregation
25	3 h	✗
25	1 d	✗
60	3 h	✗
60	1 d	✗
80	3 h	✗
80	1 d	✗

As shown in Table 3.7., for working with the concentration range between 0.05 and 5 μM, the proposed procedure is not enough. Since the serotonin levels in human blood lie within this range, 0.50-1.70 μM [50], it is important to design nanoparticles having ability to aggregate easily upon addition of serotonin. As the affinity of serotonin to sialic acid [73] was used for the sake of selectivity of the method, different modification of AuNPs was done for amine-carboxyl coupling by EDC instead of using SA-AuNPs for the coupling directly.

3.4.2. Addition of EDC/NHS to L-Cysteine to Prepare Modified Gold Nanoparticles

In order to test if there would be any improvement on binding amine group of serotonin to AuNPs, L-cys, carboxyl group containing molecule other than sialic acid, was used. After addition of L-cys solution whose carboxyl group was activated by addition of EDC/NHS to citrate reduced AuNPs, there was no change in the red color of the gold nanoparticle solution indicating no change in the particle size [73]. By mixing equal volumes of SA-AuNPs with the prepared AuNPs-L-cys+EDC/NHS solution, aggregation upon addition of serotonin was aimed. The expected reaction between AuNPs-L-cys+EDC/NHS and serotonin is shown in Figure 3.16.

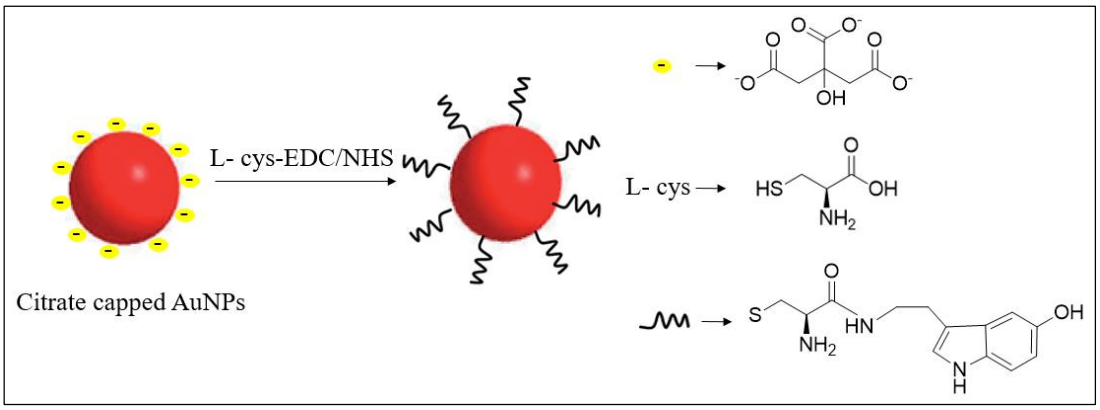


Figure 3.16. The expected reaction scheme of AuNPs-L-cys+EDC/NHS and serotonin

Table 3.8. *The reaction time and aggregation of nanoparticles upon addition of 2.5, 6.7, 12.5 and 100 µM serotonin*

Reaction Time	Aggregation
2 h	✗
4 h	✗
24 h	✓

As shown in Table 3.8., it took 1 day to observe color change in the solutions. In addition, despite of increasing the temperature to 80°C and the concentration to 100 µM, the duration time could not be shortened. In addition, it was observed that color of the AuNPs-L-cys+EDC/NHS solution itself changed color due to agglomeration of particles with time. It can be concluded that serotonin did not cause aggregation but loss of stability of particles with time did. As a result, despite of our expectation, the preparation of AuNPs-L-cys+EDC/NHS did not enhance aggregation and it was decided not to use them for the study.

3.5. Selectivity Studies

3.5.1. Selectivity Study for Sialic Acid Stabilized Gold Nanoparticles

Once a method only based on aggregation of SA-AuNPs upon serotonin addition was designed, selectivity study was performed to make sure the method can work in a

complex matrix like human blood serum. Among the molecules studied for serotonin selectivity [49], dopamine, L-cysteine, L-aspartic acid and L-glutamic acid were the available ones from the laboratory. Solutions of these molecules having 300 μM concentration were chosen since this concentration is much higher than in blood and any interference can be detected easily. Figure 3.17. shows the color of the SA-AuNPs solutions 60 min after addition of 300 μM solutions.

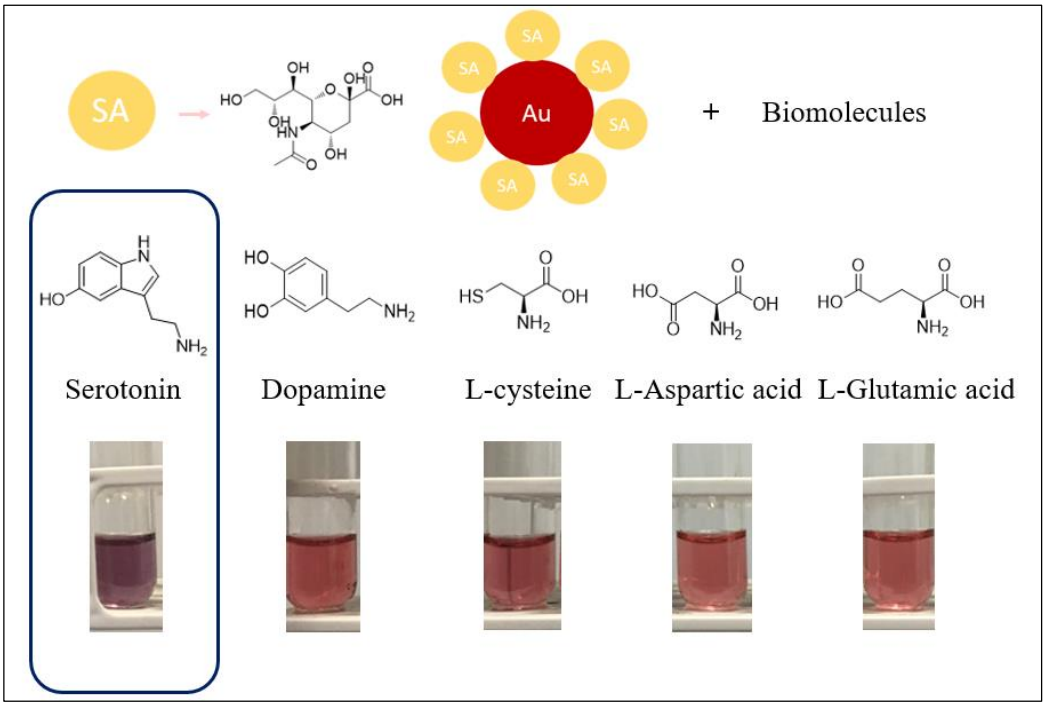


Figure 3.17. Color of the solutions after addition of 300 µM biomolecules to SA-AuNPs

Table 3.9. *A_{650} / A_{520} of biomolecules added SA-AuNPs solutions*

Added Biomolecules	**A_{650} / A_{520}**
None	0.084
Serotonin (5-HT)	0.547
Dopamine (DA)	0.098
L-cysteine (L-cys)	0.149
L-aspartic acid (AA)	0.085
L-glutamic acid (GA)	0.128

An aggregation was observed only upon addition of serotonin. Absorbance measurements are given in Table 3.9. It can be seen that the absorbance ratio (A_{650}/A_{520}) was increased significantly in serotonin addition solely, in other words a red shift in the spectrum of SA-AuNPs solution has been observed only after addition of serotonin. It is important to note that dopamine is one of most competitive molecules in serotonin determination and it showed no interference when using this developed method. Selectivity studies will be extended as other competitive molecules like epinephrine, norepinephrine, gamma-aminobutyric acid, uric acid or starting molecules in serotonin synthesis like L-tryptophan and 5-hydroxy- L-tryptophan are purchased. The selectivity of SA-AuNPs and thus the method for serotonin is supported by these results so far.

3.5.2. Selectivity Study for EDC Added Sialic Acid Stabilized Gold Nanoparticles

Selectivity of EDC+SA-AuNPs were tested by adding same biomolecules used for testing SA-AuNPs. The resulting color development 30 min after addition of 300 μM solutions is shown in the Figure 3.18.

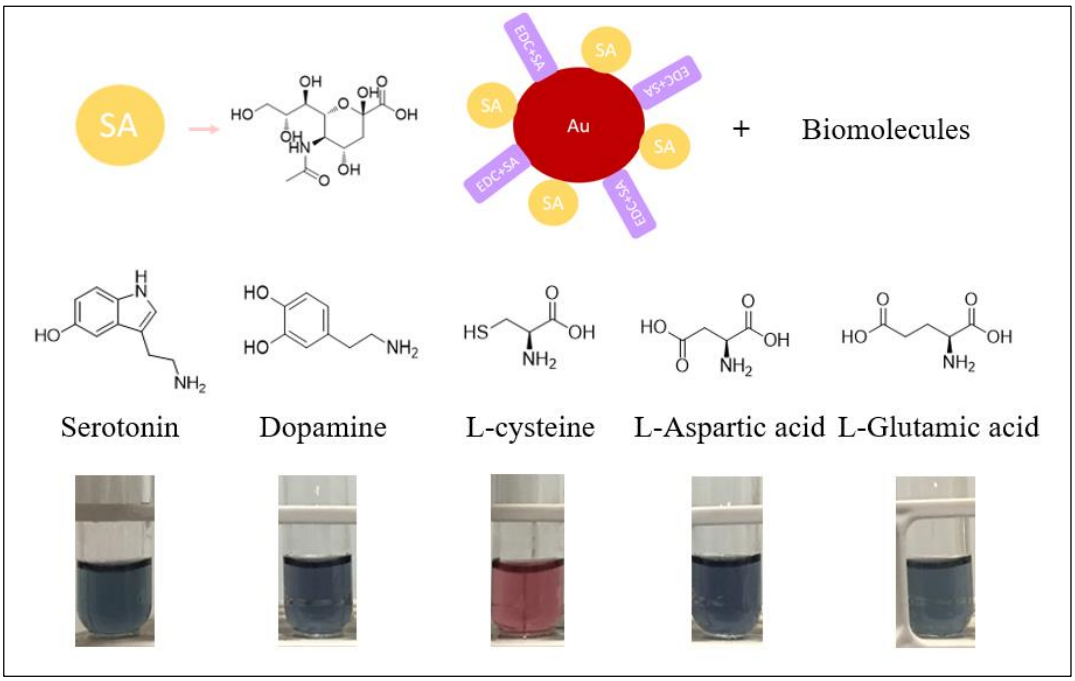


Figure 3.18. Color of the solutions after addition of 300 µM biomolecules to EDC+SA-AuNPs

Table 3.10. *A_{650} / A_{520} of biomolecules added EDC+SA-AuNPs solutions*

Added Biomolecules	**A_{650} / A_{520}**
None	0.150
Serotonin (5-HT)	1.339
Dopamine (DA)	1.322
L-cysteine (L-cys)	0.371
L-aspartic acid (AA)	1.141
L-glutamic acid (GA)	1.127

Aggregation was observed upon addition of all biomolecules tested. Absorbance measurements of gold nanoparticle solutions showing a red shift depending on the aggregation are shown in Table 3.10. The reason for the relatively low aggregation upon addition of L-cysteine might be the fact that L-cysteine contains only one –OH group whereas other molecules have two. The main reason of aggregation is that all biomolecules tested had primary amine group causing amine-carboxyl coupling with EDC added SA-AuNPs. From these results, it can be concluded that in the presence of a leaving group containing molecule like EDC attached on the surface of AuNPs, all amine group containing biomolecules can cause aggregation of nanoparticles. As a result, using EDC+SA-AuNPs in this method is not selective to serotonin and different modifications must be performed in order to work with lower concentrations of serotonin selectively.

Therefore, when the selectivity of the methods either cited in literature [49] and proposed in our study were evaluated, the binding property of the probes with the serotonin should be considered. As explained in the introduction section (pp. 9-10) the presence of acetyl group and the side chain formed from C-7, C-8 and C-9 are necessary for binding of serotonin. As shown in Figure 1.4. serotonin is captured from several functional groups of sialic acid. Thus multi-interaction of sialic acid with serotonin enhances the selectivity of sialic acid to serotonin. Consequently, the method based on the plasmonic properties of SA-AuNPs developed in our laboratory for the first time is the only selective method for the determination of serotonin.

3.6. Synthesis of Cysteamine Coated Gold Nanoparticles

In order to use only sialic acid for serotonin determination, selective side of the all sialic acid molecules on the surface of gold nanoparticles must be available. To achieve this goal, attachment of the sialic acid to AuNPs by its carboxyl group was aimed. For this reason, amine-carboxyl coupling by EDC/NHS mechanism [67] used again. However, this time, EDC/NHS was not used to bind the amine group of

serotonin. EDC/NHS mechanism was used to attach carboxyl group of sialic acid to amine group functionalized gold nanoparticles.

Red solution of AuNPs-NH_3^+ was prepared by the proposed mechanism [70] as described in the procedures section. SEM image of AuNPs-NH_3^+ and the monodispersity of the spherical nanoparticles can be seen from Figure 3.19.

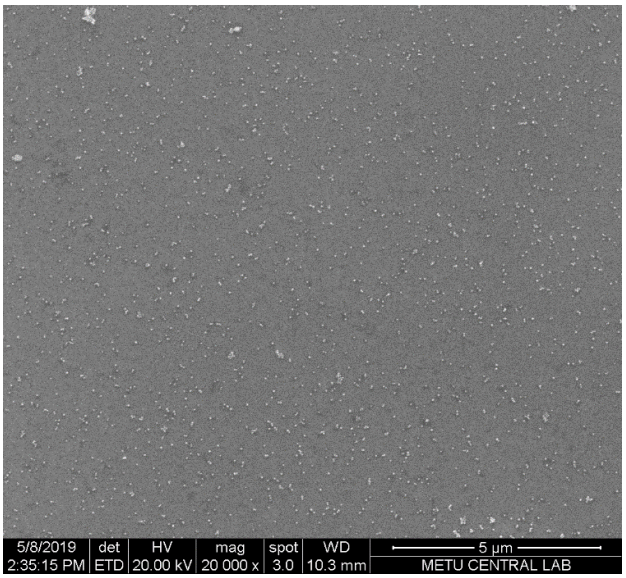


Figure 3.19. SEM image of AuNPs-NH_3^+

3.6.1. Conjugation of Carboxyl Group of Sialic Acid with AuNPs-NH_3^+

After synthesizing the amine functionalized gold nanoparticles by cysteamine, conjugation of carboxyl group containing molecule, sialic acid in our case, to AuNPs-NH_3^+ was aimed by EDC/NHS mechanism [71] proposed in page 24.

After activating the carboxyl group of sialic acid with EDC/NHS for amine conjugation, addition of SA+EDC/NHS in 0.1 M MES buffer directly to AuNPs-NH_3^+ was performed and the change in color of the solutions before and after addition is shown in the Figure 3.20.

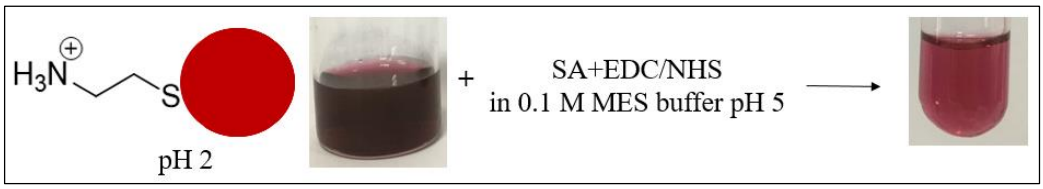


Figure 3.20. Color change of the AuNPs-NH_3^+ solution after addition of SA+EDC/NHS in 0.1 M MES buffer

Adding SA+EDC/NHS to AuNPs-NH_3^+ solution had slightly changed the color of the solution indicating increase in gold nanoparticle size [73]. Yet aggregation upon addition of serotonin can still be studied to design a colorimetric method. After dialysis of prepared nanoparticles against deionized water for 1 h, there was no aggregation even 24 h later upon addition of serotonin solutions having concentrations between 100 and 500 μM. This outcome might lead two arguments: Either the sialic acid molecules did not conjugate with the amine groups of AuNPs-NH_3^+ or the amount of sialic acid on the surface was not enough to cause aggregation.

Therefore, surface characterization of AuNPs-NH_3^+ particles after modification was done spectroscopically. FT-IR spectra of sialic acid (red line) and the nanoparticles after addition of SA+EDC/NHS to AuNPs-NH_3^+ (blue line) were taken, as shown in Figure 3.21. The realization of amine-carboxyl coupling might be followed by the presence of C=O and N-H stretchings having peaks at 1652 cm^{-1} and 3493 cm^{-1} respectively [72]. However, there was not enough evidence for the attachment of sialic on the nanoparticles by considering the FT-IR spectrum.

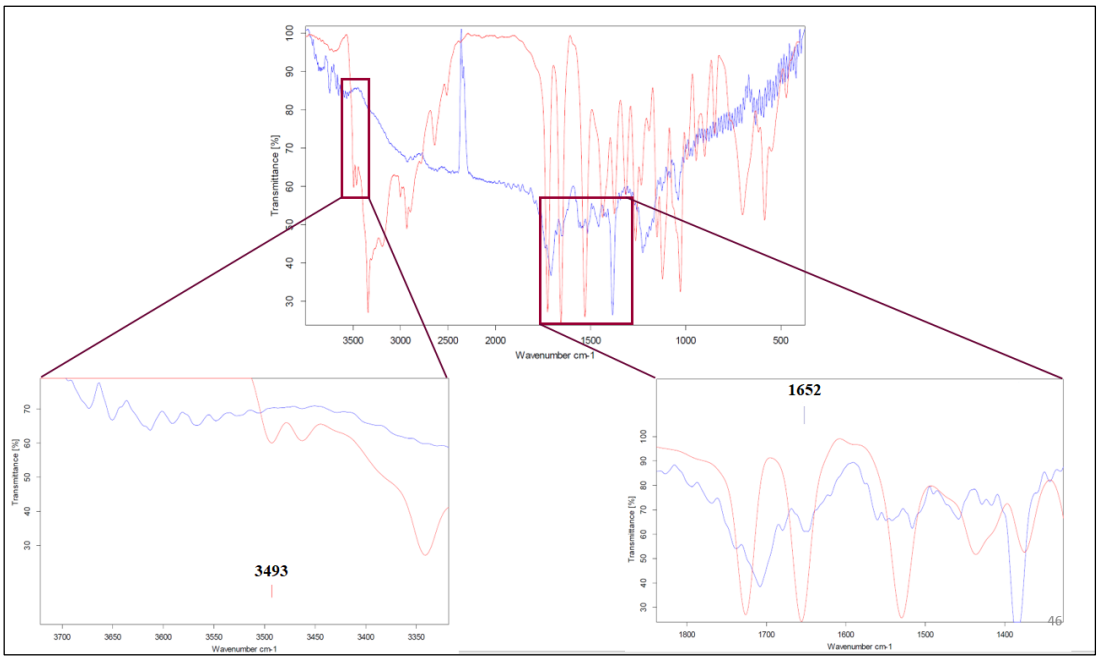


Figure 3.21. FT-IR spectra of sialic acid (red line) and the nanoparticles after addition of SA+EDC/NHS to AuNPs-NH_3^+ (blue line)

When using EDC/NHS for carboxyl-amine coupling reactions, it is important to keep pH of the solution constant although some procedures use water as the solvent [67]. MES buffer solution was used to keep pH constant when activating carboxyl group of sialic acid with EDC/NHS, however, pH adjustment for AuNPs-NH_3^+ solution was not performed and pH of solution remained as 2. This can be one of the reasons why sialic acid attachment on the cysteamine coated gold nanoparticles was not achieved sufficiently. As an optimization, pH of the AuNPs-NH_3^+ solution was increased to test if there was any enhancement in conjugation.

3.6.2. Conjugation of Carboxyl Group of Sialic Acid with AuNPs-NH_3^+ at pH 4

TEA, an organic base, was chosen to increase the pH of the AuNPs-NH_3^+ solution. After addition of TEA, the pH of the solution was 4 and the color of the solution became red-purple from red as the particles get larger due to loss of charge. Upon addition of SA+EDC/NHS in 0.1 M MES buffer directly to AuNPs-NH_3^+ at pH 4,

there was no change in particle size that can be understood from the color of the solution shown in Figure 3.22.

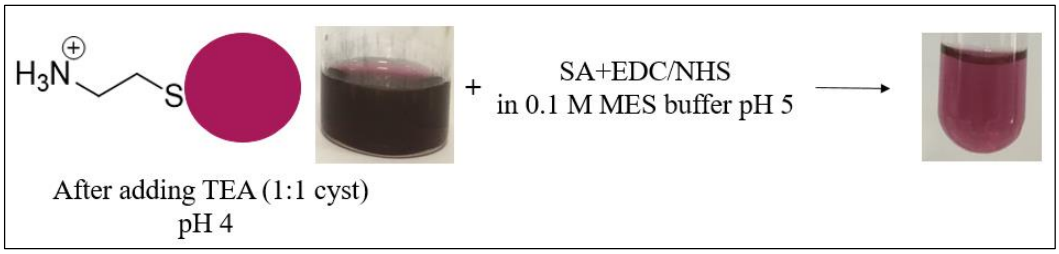


Figure 3.22. Color change of the AuNPs-NH_3^+ solution at pH 4 after addition of SA+EDC/NHS in 0.1 M MES buffer

The resulting solution was treated as the same way with AuNPs-NH_3^+ at pH 2 one. Dialyzed for 1 h against deionized water followed by addition of serotonin solutions having concentrations between 100 and 500 μM. However, despite of the bringing the pH of the AuNPs-NH_3^+ solution to 4, there was no aggregation of particles even after 24 h from addition of serotonin. This indicates any enhancement in coupling reaction between amine and carboxyl group of sialic acid was not achieved. Since TEA is an amine containing base, the interference of it to EDC/NHS coupling reaction must be considered.

3.6.3. Conjugation of Carboxyl Group of Sialic Acid with AuNPs-NH_3^+ at pH 5.5

To eliminate possible interference from any amine groups, NaOH was used to bring the pH of the AuNPs-NH_3^+ solution around the prepared MES buffer solution containing activated sialic acid. Once the pH of the solution was increased to 5.5, the color of the red solution changes as particles get larger [73] due to loss of repulsive forces between nanoparticles. The AuNPs-NH_3^+ solution at pH 5.5 was treated same as the ones at pH 2 and pH 4. Nevertheless, no color change of the solution resulting from aggregation of nanoparticles upon addition of serotonin was observed even after

24 h and increasing temperature up to 80 °C. Figure 3.23. shows the color change of AuNPs-NH_3^+ solution at pH 5.5 after addition of SA+EDC/NHS in MES buffer and serotonin respectively.

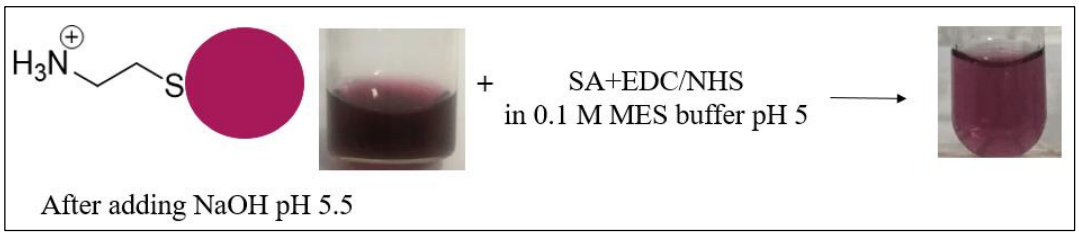


Figure 3.23. Color change of the AuNPs-NH_3^+ solution at pH 5.5 after addition of SA+EDC/NHS in 0.1 M MES buffer

CHAPTER 4

CONCLUSION

Serotonin is an important neurotransmitter and hormone which regulates many cognitive and behavioral functions. Abnormal levels of serotonin have been related with numbers of disorders and that is why it is important to determine its amount in human blood. The aim of this study is to determine the amount of serotonin with a simple, selective and sensitive colorimetric method by using the plasmonic property of gold nanoparticles.

Serotonin is known to interact with the sialic acid containing glycans, glycolipids and glycoproteins selectively. Our aim was to use that specific relation between serotonin and sialic acid for designing a chromogenic probe, AuNPs for our study, sensitive to serotonin.

As a starting point, sialic acid stabilized gold nanoparticles were synthesized. In order to optimize the amount of sialic acid attached on the surface and size of the nanoparticles, MBTH method was modified. The modified version of MBTH method was applied directly on sialic acid for the first time by this study without any former treatment. The sufficient amount of sialic acid to synthesize small nanoparticles was calculated by this method and spherical SA-AuNPs having 21 ± 3 nm size were obtained.

Serotonin solutions having different concentrations were added to prepared SA-AuNPs to monitor the color change caused by aggregation of particles. The increase of reaction temperature reduced the reaction time significantly. Therefore, when studying lower concentrations of serotonin, higher temperature was preferred. There was no color change resulting from the addition of serotonin concentration lower than

10 μM despite of increasing temperature and reaction time. As a result, 10 μM was the limit that can be measured by using only SA-AuNPs.

In order to enhance the aggregation, using not only selective side of serotonin but also primary amine group of it was aimed. To attach AuNPs to serotonin from the amine group, amine-carboxyl coupling by activating the carboxyl group with EDC was aimed. However, desired enhancement was not achieved. Using carboxyl group of a different molecule was performed to test any enhancement in aggregation. Nevertheless, EDC/NHS addition to activate the carboxyl group of L-cysteine attached AuNPs was neither practical nor useful for working with lower concentration of serotonin.

Selectivity study on synthesized AuNPs was performed to test possible interferences from other biomolecules. The study showed that SA-AuNPs are very selective for serotonin whereas the EDC added SA-AuNPs are not since all of the biomolecules tested were contained amine groups. As it was explained in results and discussion chapter, there are some studies on serotonin determination by using plasmonic property of gold nanoparticles selectively. However, from the results of this study, attaching a leaving group containing molecule to AuNPs for binding the primary amine group of serotonin results in interference of all amine group containing biomolecules. To conclude, it is not realistic to claim the selectivity of a method using a molecule can react with the amine group of serotonin. As a result, our research group regards the method developed by this study as the first selective plasmonic method for the determination of serotonin.

Another aspect for enhancement of aggregation was considered to make selective side of all sialic acid molecules available when attaching AuNPs. This time, amine containing AuNPs were synthesized first and attachment of sialic acid on the surface of these particles was aimed by EDC/NHS coupling reaction. The studies on accomplishing the attachment are continuing and as a future work.

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