DEVELOPMENT OF A BIOCHIP SYSTEM WITH MULTIPLEXING CAPABILITY FOR DETECTION OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) and MUTATIONS BASED ON SURFACE-ENHANCED RAMAN SCATTERING

by

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“this thesis is dedicated to
my family and my best friend…”

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ABSTRACT

DEVELOPMENT OF A BIOCHIP SYSTEM WITH MULTIPLEXING CAPABILITY FOR DETECTION OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) and MUTATIONS BASED ON SURFACE-ENHANCED RAMAN SCATTERING

Human genome is exposed to several types of genetic abnormalities including single nucleotide polymorphisms (SNPs), which are the most common polymorphisms throughout the human genome. Because SNPs are abundant and stable, they are widely used in diagnostics of several diseases like familial Mediterranean fever (FMF), Parkinson, and Alzheimer. Identification of SNPs provides opportunities in the diagnosis, prevention and treatment of related diseases. Current methods that are used to detect SNPs are time-consuming and expensive. Therefore, there is a need to improve the speed and reduce the cost. This can only be achieved with a technique capable of multiplexing.

Surface-enhanced Raman scattering (SERS) is a vibrational spectroscopic technique, which provides specific information about a molecule's structure due to its "fingerprinting property". The multiplexing property and high sensitivity of SERS is used to reduce the number of spots on the array chip. Considering great number of SNPs present on the human genome, achievement of the multiplex SNP detection will cut the cost and time spent for the screening SNPs.

The development of an assay for the detection of SNP mutations has been attempted based on SERS in this study. To test the feasibility of approach, M694V mutation on 10^{th} exon of MEFV gene is investigated as a model. The promising results indicate that it is possible to detect SNPs using this assay.

ÖZET

GENLERDEKİ TEK NUKLEOTİD POLİMORFİZİM (SNP) ve MUTASYONLARIN TANISI İÇİN YÜZEYDE ZENGİNLEŞTİRİLMİŞ RAMAN SAÇILMASINA (YZRS) DAYALI MULTİPLEKS ÖZELLİĞİNE SAHİP BİOÇİP GELİŞTİRİLMESİ

İnsan genomu, en yaygın polimorfizim olan tek nükleotid polimorfizimin (SNP) de içinde bulunduğu birçok genetik anomaliye maruz kalmaktadır. SNP'ler genom içerisinde çok sayıda ve kalıcı oldukları için, Akdeniz anemisi, Parkinson, Alzheimer hastalığı dahil birçok hastalığın teşhisinde yaygın bir şekilde kullanılmaktadırlar. SNP'lerin tespiti, ilgili hastalıkların teşhisinde, tedavisinde ve bu hastalıklardan korunma da önemli olanaklar sunmaktadır. SNPlerin tespitinde günümüzde kullanılan metotlar pahalı ve zaman alıcıdır. Dolayısıyla maliyeti azaltacak ve süreden tasarruf sağlayacak yöntemlerin geliştirilmesine ihtiyaç vardır. Bu da ancak multipleks özelliği olan bir yöntemle geliştirilebilecektir.

Yüzeyce zenginleştirilmiş Raman saçılması (YZRS), parmak izi (fingerprinting) özelliğinden dolayı moleküllerin yapıları hakkında bilgi veren titreşimsel spektroskopik bir tekniktir. YZRS'nin multipleks özelliği ve yüksek hassaslığı, çiplerdeki spot sayısını azaltmada kullanılmıştır. İnsan genomundaki çok sayıda SNP göz önüne alındığında multipleks olarak SNP tespitinin başarılması para ve zaman kaybının azaltılmasında önemli rol oynayacaktır.

Bu çalışmada, SNP mutasyonlarının YZRS tabanlı olarak tespitleri için bir tanı sistemi geliştirilmesi amaçlanmıştır. Yaklaşımın fizibilitesinin test edilmesi için MEFV geninin 10. ekzonunda bulunan M694V SNP mutasyonu model olarak kullanılmıştır. Elde edilen pozitif sonuçlar, geliştirilen tanı sistemi ile multipleks olarak SNP tespitinin yapılmasının mümkün olduğunu göstermiştir.

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1. INTRODUCTION

Genomes of organisms are not stable and exposed to several types of genetic abnormalities (mutations). These abnormalities include macroscopic ones such as chromosomal breakage, translocations, chromosomal deletions, duplications, etc., and microscopic abnormalities such as single nucleotide polymorphisms (SNPs), deletions and insertions of one or more nucleotides.[1] One of the microscopic abnormalities, SNPs has great potential for the identification and prevention of complex genetic diseases and pharmacokinetic research. Due to the large number of SNPs in the human genome, there is a great need for high throughput screening.

1.1. Single Nucleotide Polymorphisms

SNPs, which are the most common polymorphisms throughout the human genome, constitute 90% of the human DNA polymorphisms.[2] SNPs are single base pair changes in the genome at which more than one alternative sequences exist in individuals. SNPs may be bi, tri, and tetra-allelic, but it is a known fact that tri and tetra allelic situations are almost not seen throughout the human genome; therefore SNPs are also called "*bi-allelic markers"*. In a normal population, a single base position that is supposed to be a SNP should have at least 1% or greater frequency in a normal population[3].

Identification of more than 3 million SNP throughout v the human genome triggered the interest in the field of exploring the effects of SNPs on phenotype[4]. The localization of SNPs within genome increases their importance. Any alteration in the coding region of a gene may affect the protein function of the related gene and may be responsible for several genetic diseases and problems[5]. Therefore, SNPs are important markers for diseases, clinical genetic testing, forensics applications and determination of loss of heterozygosity. Because they are abundant and stable[6], there are a lot of SNPs that are widely used in diagnostics of several diseases such as cancers, familial Mediterranean fever (FMF), Parkinson, and Alzheimer[7-11]. The identification of these mutations could be vital for

individuals to determine their disease susceptibility and an early screening has to be in place for the preventive medicine.

1.2. Detection of SNPs

Detection of SNPs is carried out routinely for preventive diagnostics[12]. Although sequencing is the safest way of detection of SNPs, it is expensive and time consuming for clinical diagnostics. Besides, there are numerous techniques for known SNPs such as Real-time PCR, Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Denaturing High Performance Liquid Chromatography (dHPLC), Mass Spectroscopy, Pyrosequencing, Flow Cytometry, and Microarray based techniques[13-23] which are also time-consuming, and present as expensive ways of detection. Most of these techniques are also not suitable for high throughput screening. Meanwhile, the concept for current microscopic mutation identification is mostly based on microarray analysis which is the most practical one. It is carried out in two different ways; oligonucleotide probes are tagged with different fluorescence molecules and hybridized with target oligonucleotides or oligonucleotide probes are chemically arrayed on a surface and fluorescence tagged PCR fragments are hybridized to the spot[23].

Microarrays have shortcomings such as the requirement for using two lasers to scan the array, and color confusion due to incomplete hybridization. Even though, microarray based SNP screening systems can analyze many SNP regions in a short time, due to enormous number of mutation points, the cost per sample are not as low as desired. Therefore, development of a novel method that is more affordable, has a short time required application and multiplexing property in SNP detection for diagnosis of diseases is one of the significant bottlenecks to be overcome.

1.3. Familial Mediterranean Fever (FMF)

Familial Mediterranean Fever (FMF) is an autosomal recessive disease that is originated from defects in the MEFV gene which is also known as MEF. Heterogeneous mutation in MEFV gene results in autosomal dominant FMF that has same symptoms with the autosomal recessive version of disease. MEFV gene, localized in the 16p13 locus, encodes for a protein called pyrin (marenostrin) that is 781 amino acid long[24, 25].

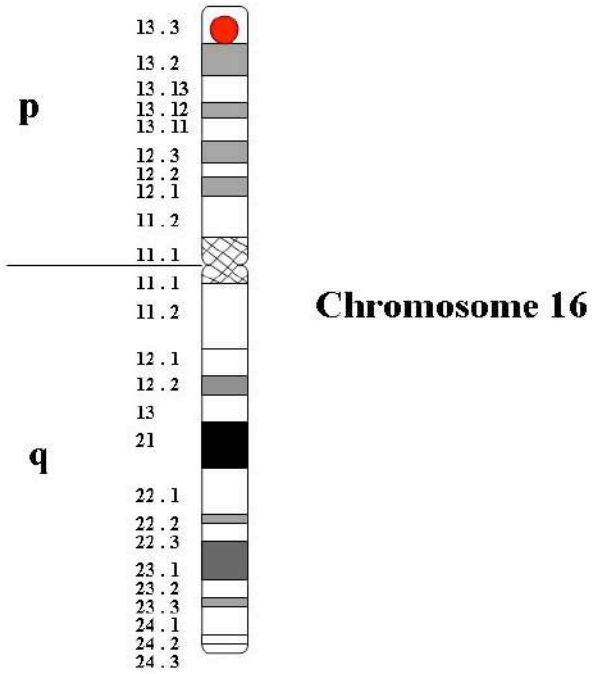


Figure 1.1 The localization of the MEFV gene in chromosome 16.

Pyrin, functioning in cytoskeleton organization, is thought to be responsible for the control of myelomonocytic cells' inflammatory response. One of the isoforms of pyrin localizes in cytoplasm and is associated with microtubules and the filamentous actin of perinuclear filaments. The other isoform is localized in the nucleus. MEFV gene is expressed in peripheral blood leukocytes and partially in mature granulocytes and monocytes[26].

FMF is a hereditary periodic fever syndrome that is known with recurrent attacks of fever, serosal inflammation, pain in the abdomen, chest or joints. FMF is mostly seen around the Mediterranean basin among the ancestral ethnic groups including Turks, Arabs, North African Jews and Armenians[27].

MEFV gene is composed of 10 exons. There are more than thirty SNP mutations that are associated with FMF (Figure 1.2).

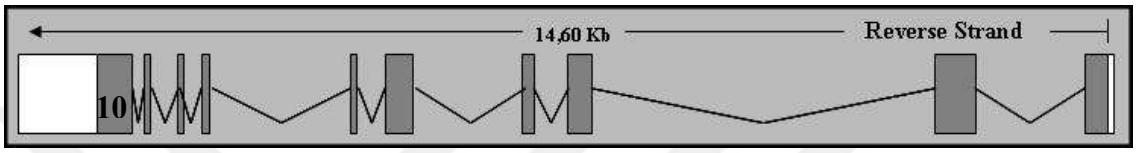


Figure 1.2 M694V mutation is seen in the 10th exon of MEFV gene.

M694V is the most common mutation among the FMF patients and M694V/M694V is the most prevalent genotype[28]. M694V mutation is used in this study to develop and test the feasibility of a microchip system to detect mutations in a single spot with utilization of nanotechnology and surface enhanced Raman Scattering (SERS).

1.4. Raman Spectroscopy: A Vibrational Spectroscopic Technique

Raman spectroscopy is a vibrational spectroscopic technique that relies on Raman scattering (inelastic scattering) of monochromatic light such as a laser in the visible, near infrared and near ultraviolet range. Raman effect is seen when laser beam interacts with the electron cloud and the bonds of a molecule which results in the change of molecular polarization potential, the amount of deformation of the electron cloud, with respect to the vibrational coordinate. The amount of the change in the polarizability gives the intensity of Raman scattering. Raman Spectroscopy is mostly used in chemistry and gives fingerprint information about the chemical bonds in a molecule.[29]

Raman spectroscopy has several advantages over microscopic analysis. Because Raman spectroscopy is a scattering technique, there is no need to fix or section the specimens and spectra can be collected from a very small amount of sample.[30] Most

importantly, Raman has immunity to water which means Raman spectral analysis isn't affected from water content of the sample that is very important in analysis of biological samples.[31]

Meanwhile, Raman scattering is very weak, and main difficulty about Raman spectroscopy is to differentiate the inelastic Raman scattering from the intense Rayleigh scattering. Also, low intensity of the collected spectrum, and the long acquisition duration are other important disadvantages of Raman spectroscopy. There are a variety of Raman spectroscopy types such as surface-enhanced Raman, tip-enhanced Raman, polarized Raman, stimulated Raman, transmission Raman, spatially-offset Raman, and hyper Raman. The leading purposes to advance the Raman spectroscopy are enhancing the sensitivity, improving the spatial resolution, and acquiring very specific information. The technique used in this study, surface-enhanced Raman scattering mostly aims to enhance the Raman scattering intensity.[29]

1.5. Nanotechnology, Gold and Silver Nanoparticles and SERS

Nanotechnology is the creation and application of materials, devices, and systems with utilization of nanometer-sized structures[32]. Several fields such as biology, physics, chemistry, and electronics conduct interdisciplinary studies in nanotechnology to understand the behavior of nanometer scale structures for their possible novel applications. In biomedical sciences and medicine, nanotechnology is utilized to develop assays for applications such as delivery, diagnosis, treatment and imaging. Preparation of nanoparticles enhanced the progression in nanotechnology, and noble metal nanoparticles, especially silver and gold nanoparticles with unique physicochemical properties found a significant use in different nanobiotechnology applications[33-36].

Having extraordinary optical properties and being biocompatible and non-toxic to living cells, Gold Nanoparticles (GNPs) are prevalently and preferentially used in biomedical studies. As a result of interaction of light with noble metal nanoparticles such as gold and silver nanoparticles, a unique optical property, formation of plasmons is observed during the interaction of electromagnetic fields of light with the conductor metal's oscillating electron plasma[37]. (Figure 1.3)

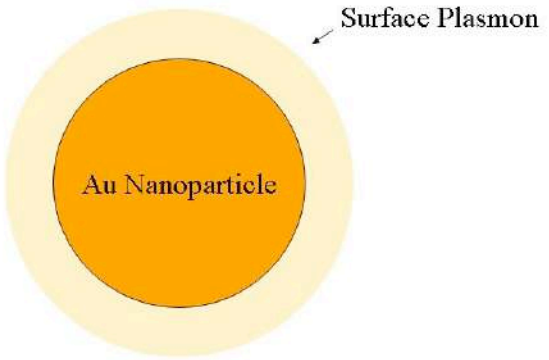


Figure 1.3 Localized surface plasmons (LSPR)

One of the significant implications of the surface plasmons is the enhancement in Raman scattering. When a molecule or a molecular structure is brought into close contact with the noble metal nanostructures, an extraordinary enhancement in Raman scattering, up to 10^{14} times, is observed. This phenomenon is called surface-enhanced Raman scattering (SERS). SERS is a vibrational spectroscopic technique, which provides specific information about a molecule's structure and this information is considered as the "fingerprinting" of the molecule. It provides detailed information about the functional groups rich in electron in a given molecular structure. The obtained SERS spectra contain very narrow and highly resolved bandwidths (naturally less than 1 nm) compared to the fluorescence bandwidths (50-100 nm for fluorescing molecules) and provide detailed chemical information about the molecular structure.[38-42]. SERS spectra of some sample dyes are shown in Figure 1.4.

Although Raman scattering is weak because of its nature, along with the enhancement of the Raman signal intensity as a result of the excitation of surface plasmons of noble metal nanoparticles, recent developments in laser and detector technology have increased the sensitivity of SERS to the levels of fluorescence spectroscopy technology[43-45]. It was demonstrated that adsorption of molecules to gold or silver surfaces or nanoparticles enhances the Raman scattering 10^4-10^6 times and in some certain

circumstances enhancement reaches up to 10^{14} times making detection of even a single molecule possible[46].

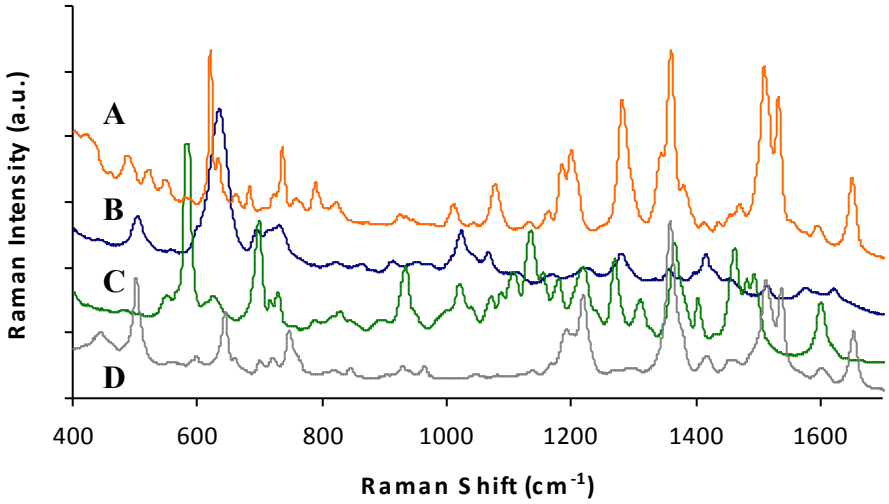


Figure 1.4 SERS spectrum of Rhodamine B (A), Cy 3,5 (B), Cy 5 (C), and TAMRA (D) dyes

1.6. The Multiplexing Property

Along with its high sensitivity and non-destructive feature, SERS is a suitable technique for multiplex detection.[47, 48] The multiplex detection requires more than one molecule that provides distinguishable signal coming from each molecule upon their overlapping signals. This means that multiple Raman spectra can be combined without significant overlap. There are techniques such as fluorescence, which can offer multiplexing capability. However, the extensive overlapping of spectral bands of fluorescing molecules could be a significant problem. Therefore, all microarray screening techniques that are based on fluorescence detection are less desirable for multiplexing.

There are several reports demonstrating the utility of the SERS based mutation detection using the multiplexing property[49-55]. These developed "DNA microarray assays" based on Raman scattering have several advantages over the assays based on fluorescence detection[50]; Raman signal is collected using a single laser and the number of Raman active dyes is more than fluorescence dyes. Besides, small modifications on Raman active dyes

result in a molecule that have different Raman signal which is not possible with fluorescence molecules[56,57]. It is indeed the derivatives of the Rhodamine dyes used in this study (Figure 1.5).

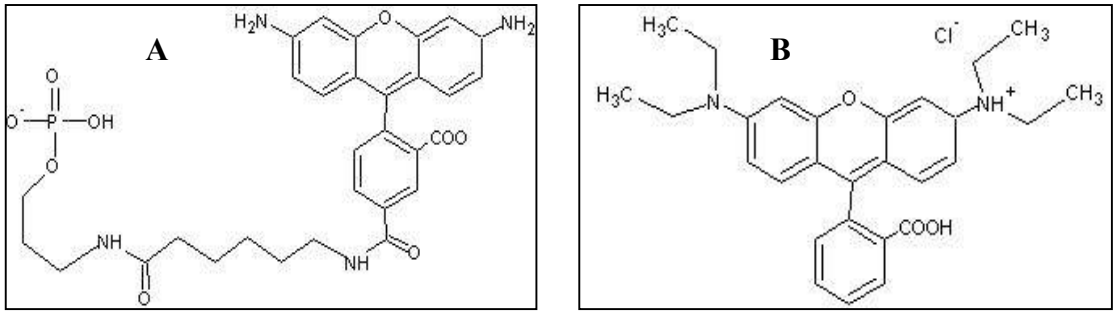


Figure 1.5 Chemical structure of TAMRA (A) and Rhodamine B (B) dyes which are derivatives of Rhodamine.

When more than one SNP region is amplified and immobilized on a single spot, with design of additional probes, it is possible to identify more SNPs on the same spot without extra effort. With an intelligent dye choice, detection of several SNPs with single Raman measurement on the same spot is feasible. The possibility of detecting multiple species allows the reduction of the number of spots on the array chip, and, for example, more SNPs can be screened on the same given area of a fluorescence-based micro-array. Considering large number of SNPs present on the human genome, achievement of the multiplex SNP detection will cut the cost and time spent for the screening SNPs.

The development of an assay for the detection of gene mutations has been attempted based on SERS in this study. M694V mutation on 10th exon of MEFV gene is investigated as a model, to test the feasibility of this approach. The PCR amplified DNA is immobilized on the poly-L-Lysine coated glass surface after denaturing with heating. The SERS probes are prepared by simultaneous attachment of oligonucleotide probes complementary to the target mutation regions and Raman active dyes to 13 nm GNPs. After the hybridization of SERS probes on the poly-L-Lysine surfaces, the array was stained with silver colloidal nanoparticles for further enhancement of Raman scattering.

2. MATERIALS and METHODS

2.1 Reagents

Oligonucleotide primers and oligonucleotide probes were purchased from Operon Technologies. $HAuCl_4 \cdot 3H_2O$, $AgNO_3$, Cysteamine, TAMRA are purchased from Fluka (Taufkirchen, Germany). Poly-L-Lysine, Cysteine, DMF, Lysine, and dNTP are purchased from Sigma-Aldrich (Taufkirchen, Germany). Sodium Citrate is purchased from Merck (New Jersey, USA). Rhodamine B, TSTU, and DIPEA are purchased from Alfa Aesar (Karlsruhe, Germany). Acetonitrile is purchased from LabKim (Istanbul, Turkey). "PCR set" is purchased from Fermentas (Maryland, USA).

2.2 Oligonucleotides used for Mutation Detection

Oligonucleotides are designed with a free thiol group on one end of the sequence in order to attach oligonucleotides to GNPs. For detection of 2121(M694V) variation on MEFV gene, TAATGATGAAGG-AAAAAAAAAA-$(CH_2)_6$SH was used as wild type probe and TAATGCTGAAGG-AAAAAAAAAA-$(CH_2)_6$SH was used as mutant probe.

2.3 Synthesis of Gold Nanoparticles

GNPs were prepared by reduction of $HAuCl_4 \cdot 3H_2O$ with sodium citrate. This procedure generates an average size of 13 nm GNPs that are well characterized in the literature[58]. Figure 2.2 and 2.1 show the TEM image and UV-Vis spectroscopic analysis of GNPs synthesized as described here, respectively. Briefly, a 50 ml of 38,8 mM citrate stock solution was added into 500 ml, 1mM of $HAuCl_4 \cdot 3H_2O$ solution. The HAuCl4$\cdot 3H_2O$ solution was heated until to boil, and then the citrate solution was added into the boiling solution. The final solution was kept boiling for 15 min.

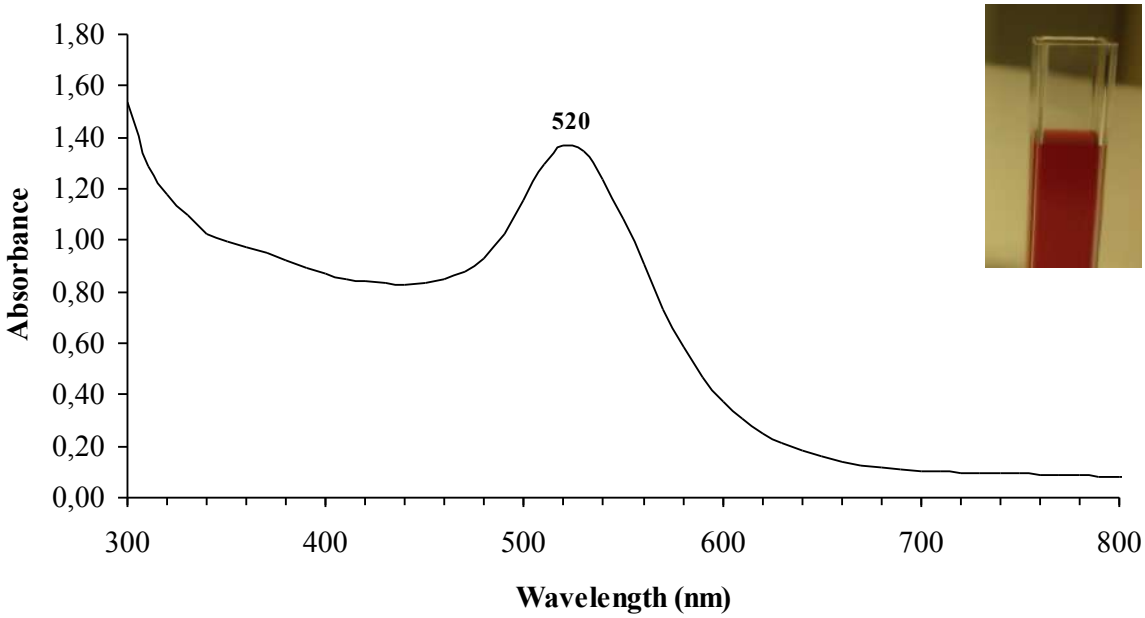


Figure 2.1 Characterization of 13 nm GNPs with UV-Vis Spectroscopy

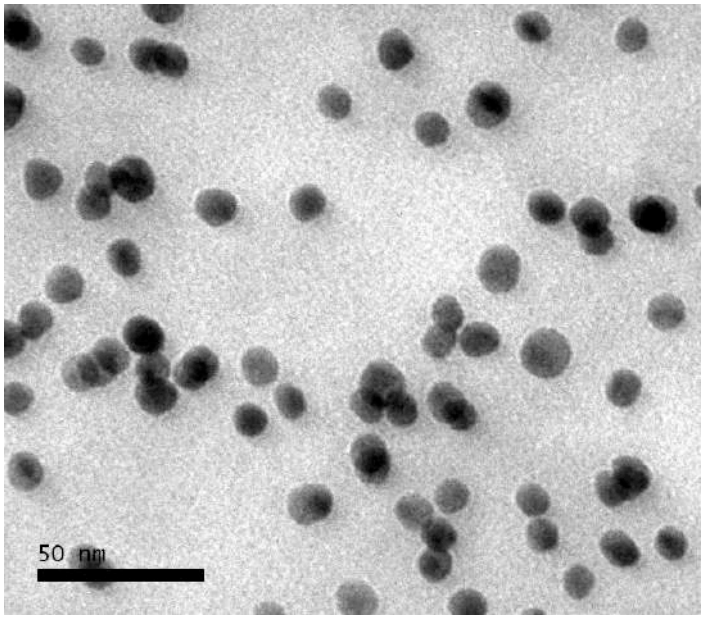


Figure 2.2 TEM image of citrate reduced GNPs

2.4 Synthesis of Silver Nanoparticles

Silver colloid was prepared according to the method reported by Lee and Meisel[59]. Briefly, 90 mg $AgNO_3$ was dissolved in 500 mL water. This solution was heated until it boiled. A 10 mL aliquot of 1% sodium citrate was added into the solution, heating was maintained approximately for 1 h until the volume of remained solution reached half of the initial volume. The absorption maximum of silver colloid solution was recorded at 420 nm. Figure 2.3 shows the UV-Vis spectroscopic analysis of GNPs synthesized as described here.

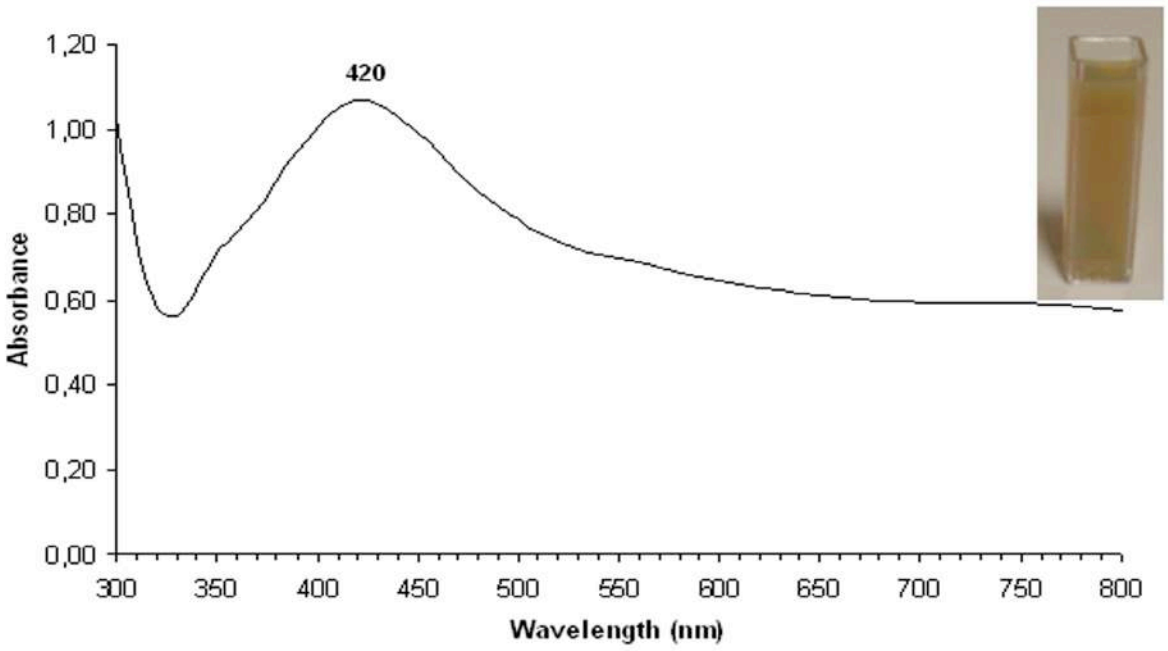


Figure 2.3 Characterization of Silver Nanoparticles with UV-Vis Spectroscopy

2.5 Preparation of SERS Probes

The GNPs with an average size of 13 nm were derivatized with Raman active dye molecules and thiol attached oligonucleotides simultaneously. The GNPs were modified with the Raman active dyes and thiolated single strand DNA, which is complementary to the target DNA.

2.5.1 Activation of dyes

The dyes that have carboxyl groups were activated to make the dyes become ready for attachment of amine groups of the target molecules[60]. *O*-(*N*-succinimidyl)-*N*,*N*,*N*,*N*-tetramethyluronium tetrafluoroborate (TSTU) and N,N-Diisopropylethylamine (DIPEA) were used to activate the dyes in different organic solvents such as acetonitrile (Method I) and DMF (Method II).

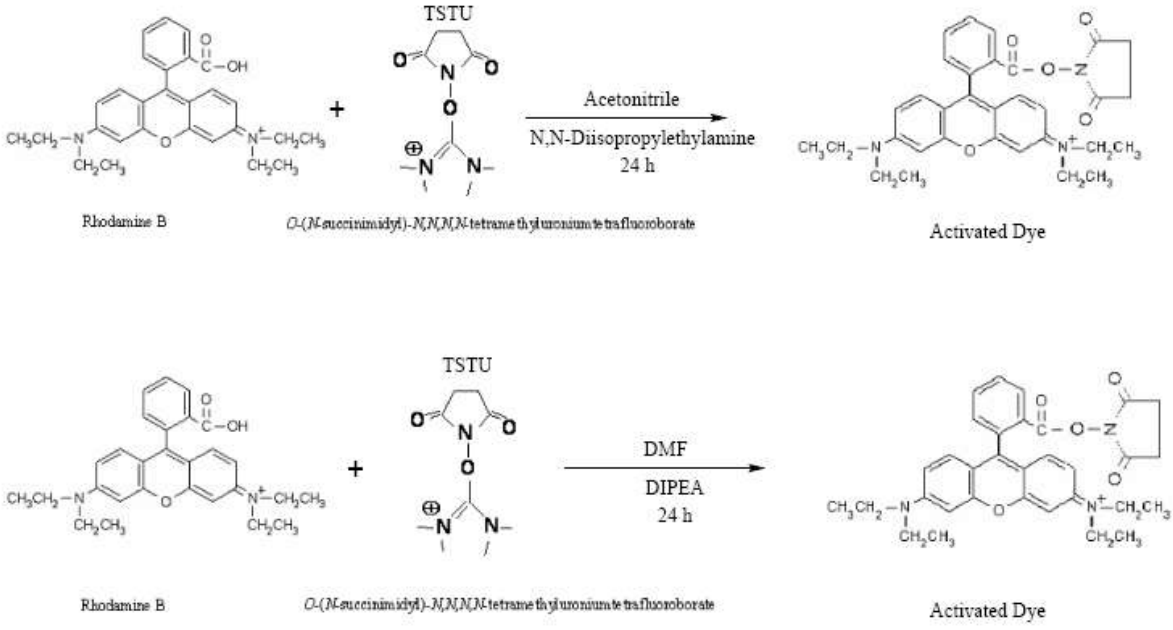


Figure 2.4 Activation of Dyes

2.5.1.1 Activation of Dyes in Acetonitrile

To have dye solutions in similar concentrations, 2,4 mg of Rhodamine B (479,02 gr/mol; ~ 0,05 M) and 2,0 mg of TAMRA (430,46 gr/mol; ~ 0,05 M) dyes were dissolved in 9 ml of acetonitrile solution. 2,4 mg TSTU (activator, M_A= 301.05 gr/mol ; 8 x 10^{-6} mol) and 150 µL, 5,8 M (118,8 µL DIPEA + 31,2 µL Acetonitrile,)* N,N-Diisoproplamine (DIPEA) were added to the dye solutions. The mixtures were left for 24 hours for incubation to complete the activation process (Figure 2.4).

* A DIPEA stock solution which has 7,3 M concentration was used. According to the procedure followed, 150 µL, 5,8 M DIPEA solution was needed. Therefore 118,8 µL DIPEA from stock solution is completed to 150 µL with acetonitrile to have the desired concentration and volume (Figure 2.4).

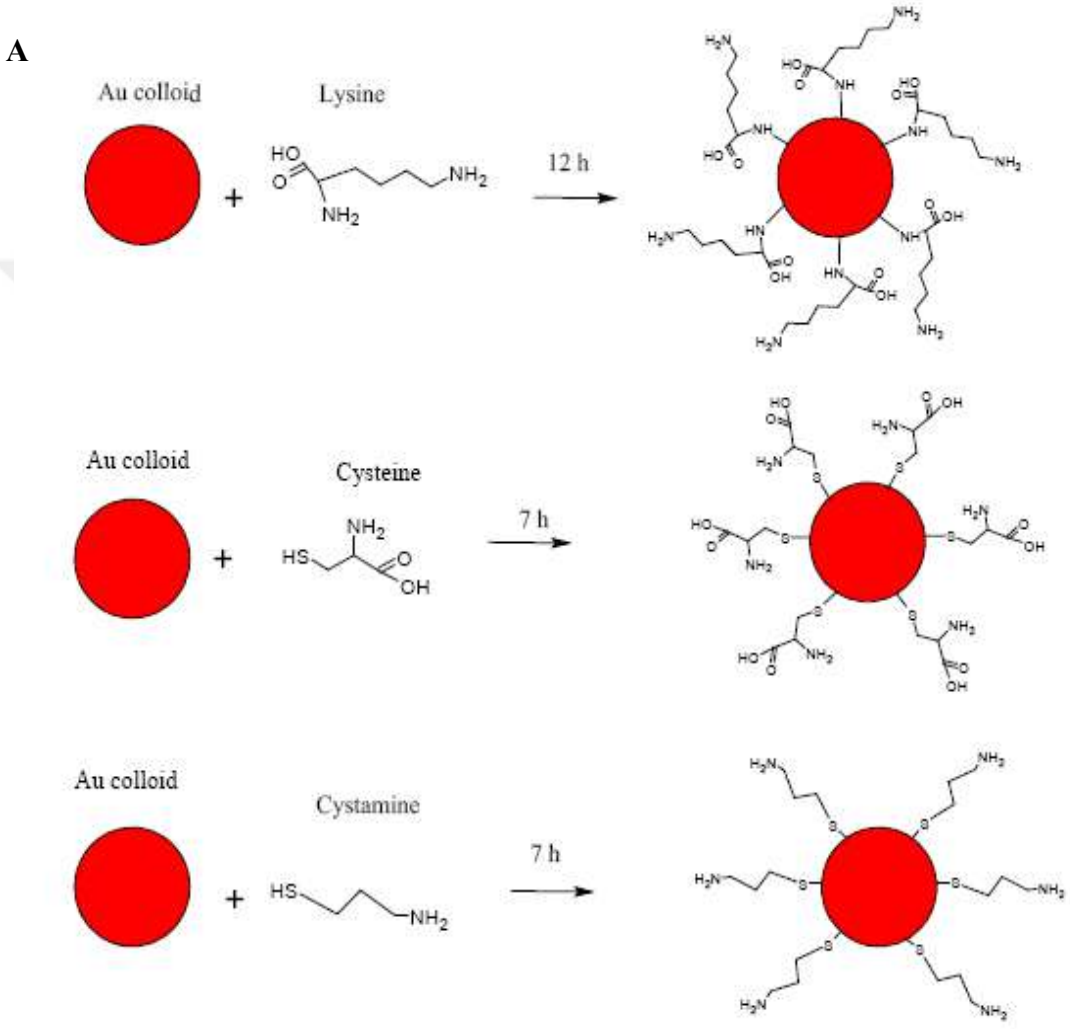
2.5.1.2 Activation of Dyes in DMF

In the second method of activation, 1x dye, 1,2x TSTU [0,0361 gr to have 0,012 M final concentration] and DIPEA [16,41 µL from stock solution to have 0,012 M final concentration] were added into 10 ml DMF. 1x dye corresponds to 0,01 M of each dyes; Rhodamine B (0,048 gr) and TAMRA (0,043 gr). The mixtures were left incubation at room temperature for 24 hours to complete the activation process (Figure 2.4).

2.5.2 Attachment of Spacer Molecules

Lysine (Ma = 146.19 g/mol), cysteine (Ma = 121.16 g/mol), and cysteamine (Ma = 113.61 g/mol) as alternative spacers and two different methods were used to accomplish the attachment of dyes to the GNPs. In the first method, the spacer molecules are chemically attached to the surface of gold nanoparticles through their thiol groups and then the activated dyes were chemically attached to the free amine groups of spacer molecules. The concentrations of spacer molecules were arranged to be 10^{-3} M in the final concentration. 100 µl of spacer molecules in 10^{-3} M concentration were incubated with 900 µl of GNP solution. Since GNPs formed aggregates after incubation process, dye attachment step couldn't be performed. The first method is shown in Figure 2.5.

A


Au colloid
Lysine
12 h
Au colloid
Cysteine
7 h
Au colloid
Cystamine
7 h

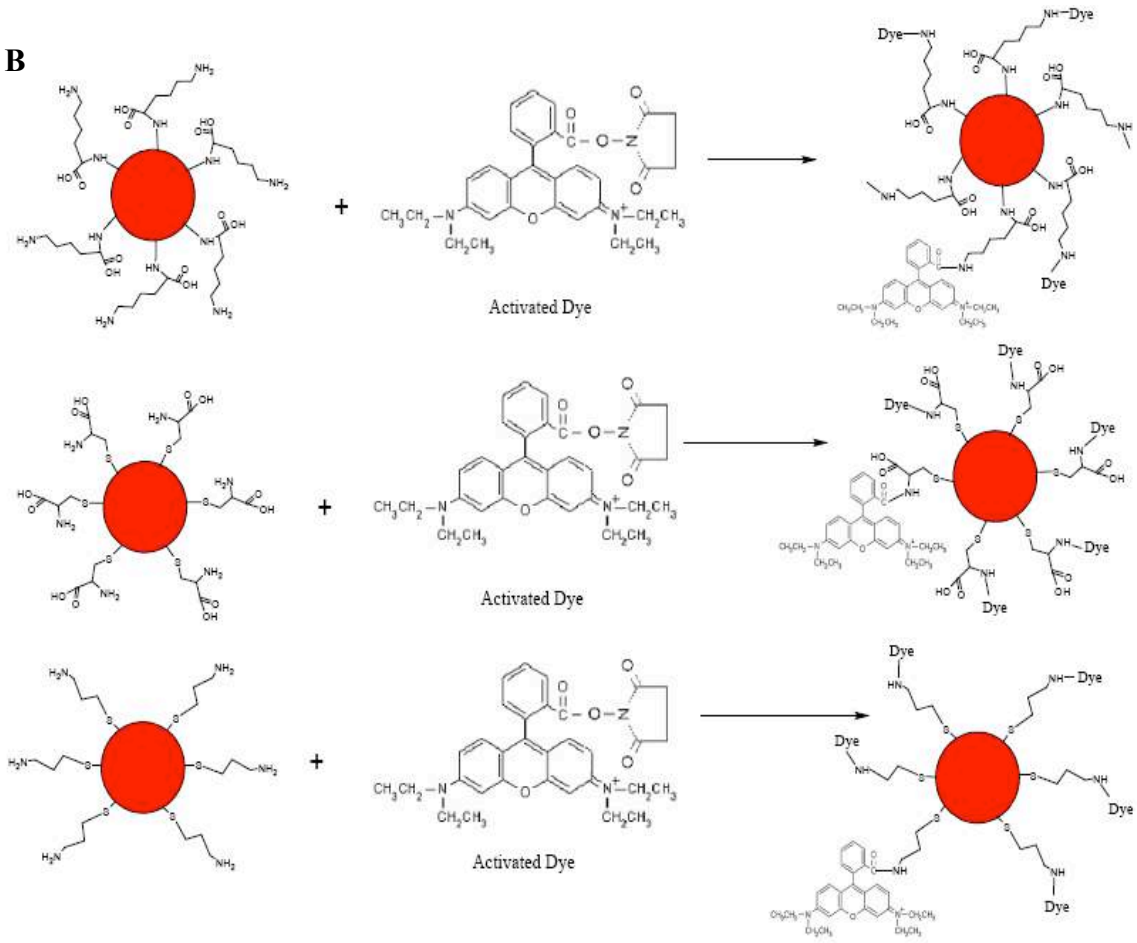
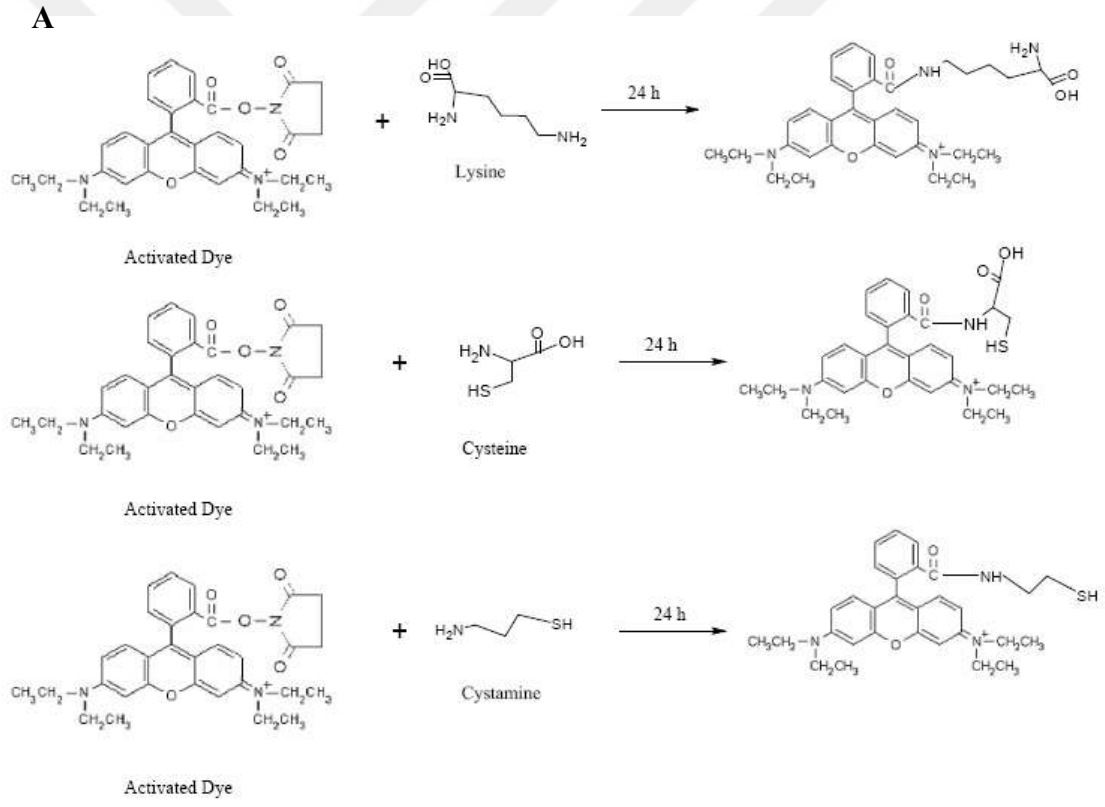


Figure 2.5 The modification of surfaces of GNPs with spacer molecules (A) and chemical attachment of the activated dyes to the spacer modified GNPs (B)

In the second method, as a first step, activated dyes were modified with the spacer molecules and then mixed with the GNPs to form dye modified GNPs. 900 µl of activated dyes were incubated with 100 µl of spacer molecule solutions in 10^{-3} M concentration. After incubation of 24 hours at room temperature, 90 µl of spacer molecule attached dyes were left for incubation with 1 ml of GNP solution 7 to 12 hours at room temperature. (7 hours for lysine and cysteine, 12 hours for cysteamine). This method is demonstrated in Figure 2.6.



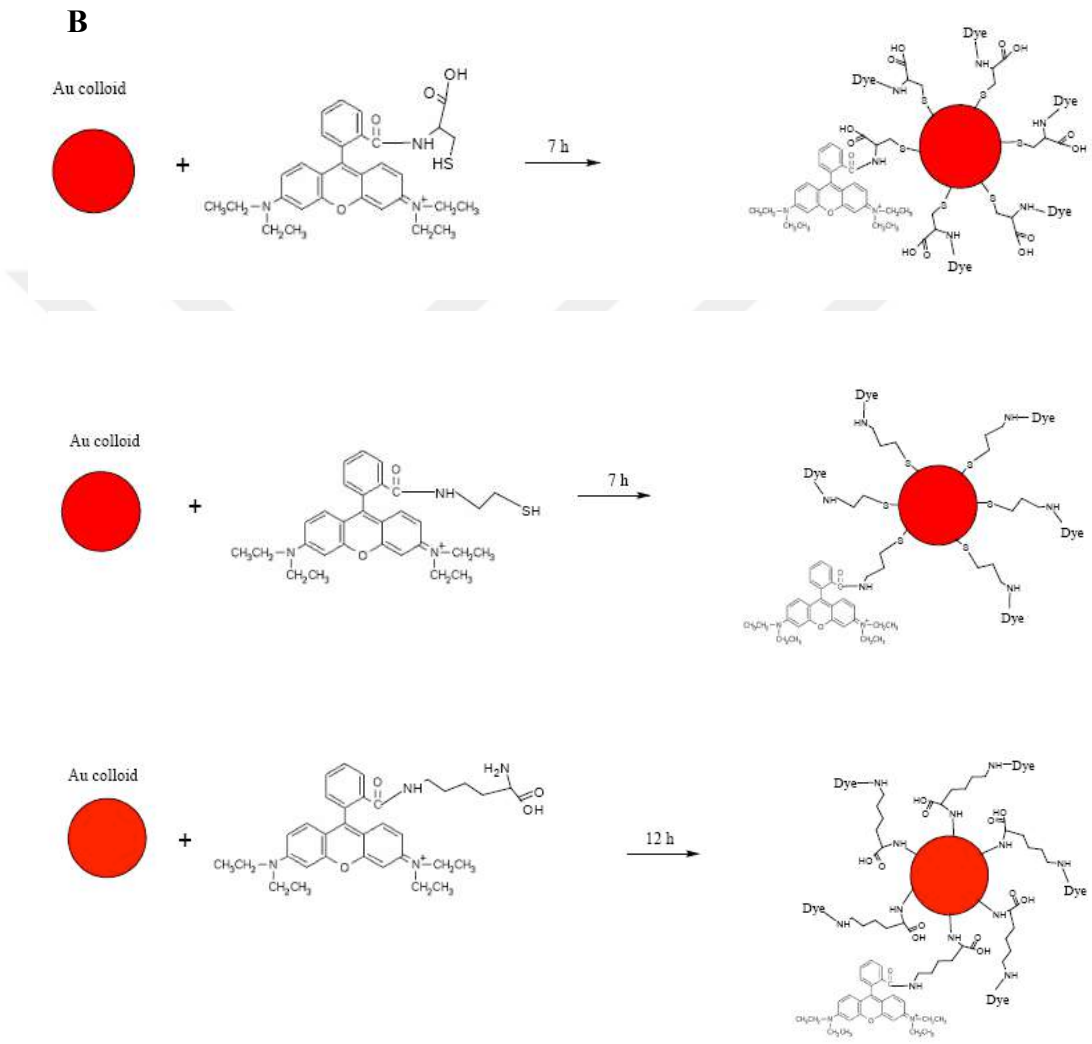


Figure 2.6 The interaction of spacer molecules with activated dyes (A) and attachment of spacer molecule modified activated dyes to the GNPs

2.5.3 Binding of Dyes and Oligonucleotides to GNPs

Activation of dyes in acetonitrile and attachment of spacer molecules firstly to the activated dyes were found to be more efficient than the alternative methods. Cysteamine was chosen as spacer molecule.

2.5.3.1 Preparation of Probes with Modified Dyes

There are approximately 10^{12} gold nanoparticles in 1 ml of synthesized GNP solution. Nearly 100 activated and spacer molecule attached dye molecules can bind to the surface of a single GNP. Therefore 10^{14} activated and spacer molecule attached dye molecules are needed to modify 1 ml of GNP solution completely. Because chemical reactions cannot occur 100% efficiently and unbound dyes can be removed upon centrifugation, 90 μL of dye solutions containing 10^{16} dye molecules are added to 1 ml of GNP solution. Figure 2.7 shows the summary of dye attachment procedure. The mixtures were left incubation at room temperature for at least 12 hours to complete the attachment process.

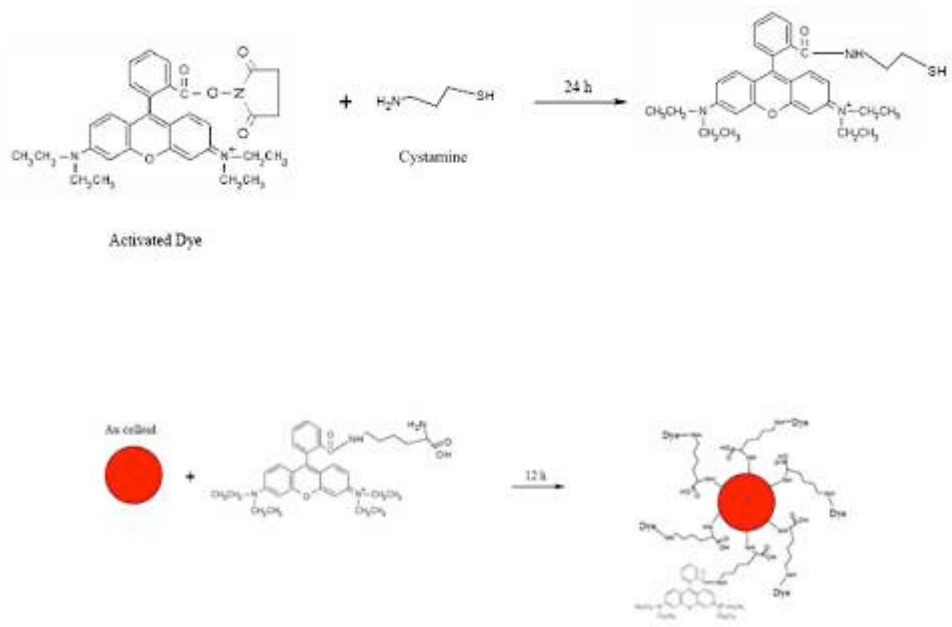


Figure 2.7 The summary of chemical attachment of Raman active dye derivatized with a thiol group, onto the surfaces of GNPs. 900 µl of activated dye and 100 µl of 10^{-3} M cysteamine were incubated together for 24 hours at room temperature. Then 90 µl of spacer molecule bound dye solution was incubated with 1 ml of GNP solution for at least 12 hours at room temperature to have dye attached GNP probes.

2.5.3.2 Preparation of Probes with both Modified Dyes and Oligonucleotides

The modified dyes and single strand DNA were simultaneously attached to the gold nanoparticles surface to prepare SERS probes. In addition to 90 µL of dye solutions, 100 µM, 17 µl thiol modified oligonucleotide solutions that contain 10^{15} single oligonucleotide strand were added to 1 ml of GNP solution. Figure 2.8 shows the summary of the attachment of both dye and oligonucleotide. The mixtures were left incubation for at least 12 hours to complete the attachment process.

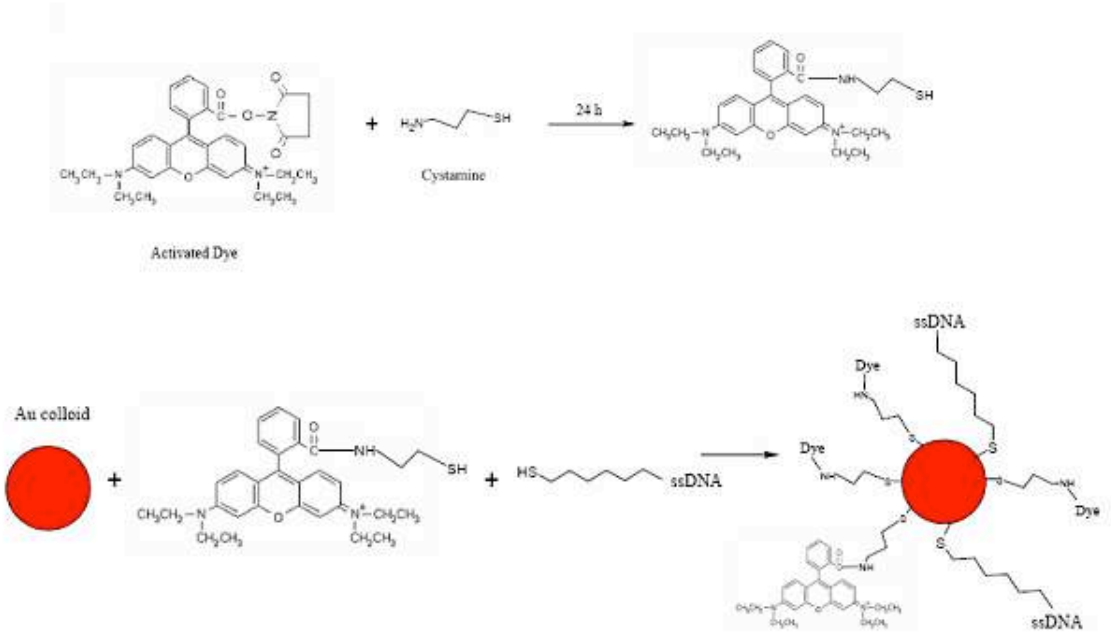


Figure 2.8 The summary of chemical attachment of oligonucleotide and Raman active dye derivatized with a thiol group onto the surfaces of GNPs. 900 µl of activated dye and 100 µl of 10^{-3} M cysteamine were incubated together for 24 hours at room temperature. Then 90 µl of spacer molecule bound dye solution and 17 µl of oligonucleotide solution were incubated with 1 ml of GNP solution for at least 12 hours at room temperature to have dye attached GNP probes.

After enough time for incubation, probes were washed in three steps for effective removal of excess dye and oligonucleotide molecules. Pellets were washed with 1 ml of distilled water after each centrifugation. After last centrifugation pellets were dissolved in 0,1 M $NaNO_3$, 10 mM Phosphate solution (pH 7).

Washing steps were as following;

- 1^{st} Wash Step, 10000 rpm, 30 minutes
  Vigorous vortexing and sonication at room temperature for 30 minutes
- 2^{nd} Wash Step, 7500 rpm, 20 minutes
  Vigorous vortexing and sonication at room temperature for 30 minutes
- 3^{rd} Wash Step, 7500 rpm, 20 minutes
  Vigorous vortexing and sonication at room temperature for 30 minutes

Preparation of SERS probes was completed after washing steps. Then 5 µL from each probe solution was dropped on a CaF_2 slide and left to dry and then 5 µL of Ag nanoparticle solution was dropped on each spot. Upon drying of spots in a laminar flow, SERS activity of probes was analyzed with Raman Microscopy.

2.6 Isolation of Genomic DNA

Blood samples were kindly provided in EDTA vacutainer tubes by Yeditepe University Hospital Genetics Diagnosis Center with the consents of patients. Peripheral blood samples were centrifuged at 4000 rpm for 15 minutes and buffy coats were transferred to a new tube to isolate genomic DNA using "Qiagen Mini Blood Kit" according to manufacturer's procedure. DNA isolation was performed from both people who are carriers of mutations and from ones that carry wild type allele. The purity and concentration of DNA samples were analyzed with nanodrop UV spectrometry (Implen GmBH).

2.7 PCR conditions

PCR reagents and conditions were optimized prior to amplification of intended fragment. PCR reactions were carried out using 5'-GAGAATGGCTACTGGTGGTG-3' primer as sense and 5'-GCGAATGTATAGATGTGGGA-3' primer as antisense primer for amplifying a 178 bp fragment containing 2121 (M694V) mutation in the exon 10 of the MEFV gene (locus: MN00243). The reactions were performed using 38 cycles consisting of 95°C for 1 min, 52°C for 50 sec and 72°C for 50 sec. PCR products were run on 2% agarose gel for 30 minutes at 100 V.

2.8 Isolation of Amplified DNA Fragments from Agarose Gel

Amplified DNA fragments from agarose gel were isolated using "Promega Wizard SV Gel and PCR Clean-UP System". PCR products were run on 2% agarose gels and DNA bands were cut out of gel with scalpels and DNA fragments were isolated according to the manufacturer's instructions. A small amount of isolated DNA fragments were rerun on

agarose gel to confirm the isolation process. The purity and concentration of DNA samples were analyzed with nanodrop UV spectrometry (Implen GmBH).

2.9 Preparation of Poly-L-Lysine slides

Glass slides that will be coated with poly-L-Lysine were left in a 2N NaOH solution for 2 hours. Then slides were washed with 200 ml distilled water 5 times and they were left in 0,01% poly-L-lysine solution for an hour in plastic wares. After all poly-L-Lysine coated slides were washed with 200 ml distilled water and 200 ml 100% ethanol, they were left for drying in laminar flow. The AFM image of poly-L-Lysine slides is demonstrated in Figure 2.9.

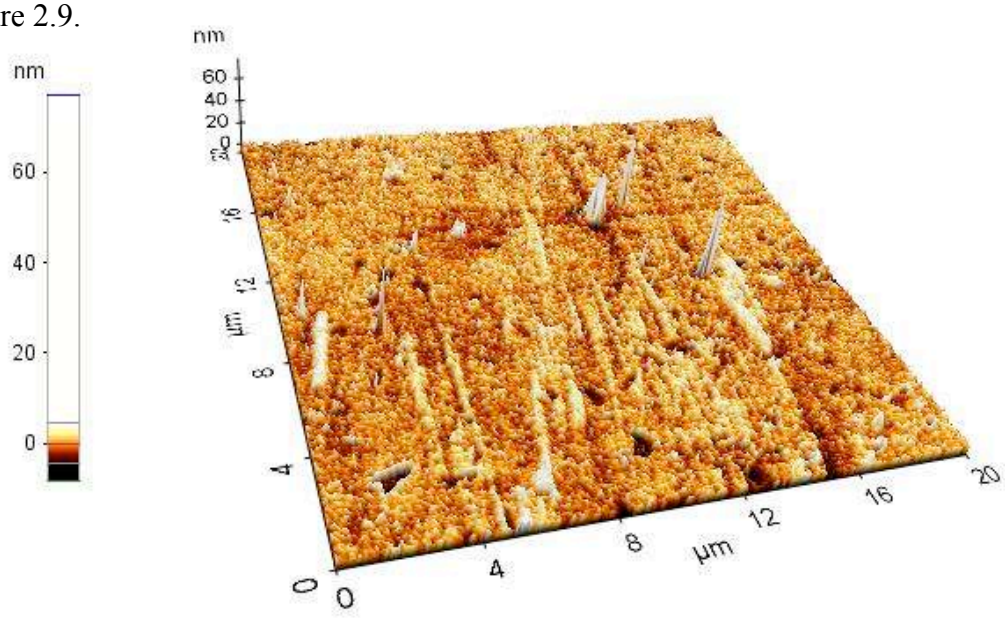


Figure 2.9 The AFM image of poly-L-Lysine coated glass slide

2.10 Spotting DNA on Poly-L-Lysine slides

DNA fragments isolated from agarose gel were heat denatured at 95°C for 5 minutes and immediately put in an ice bath at 0°C to make force DNA stay in single stranded structure. The single stranded DNA was spotted on poly-L-Lysine coated slides and chemically cross-linked to the surface with a UV crosslinker (Figure 2.10 and 2.11).

Figure 2.10 UV Cross-linker

The slides were vigorously washed with distilled water to remove unbound DNA from the slide surface as a final step in preparing DNA spots. Optimization of spotting was carried out by changing the spotted DNA amount and UV energy level exposed.

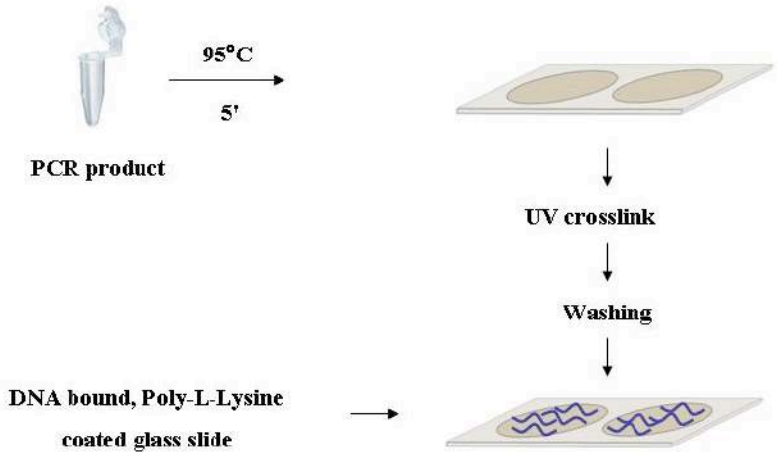


Figure 2.11 Coating of Poly-L-Lysine slides with DNA

2.11 Hybridization of Probes to the Genomic DNA

An appropriate amount from each probe was dropped manually on the genomic DNA spots for detection of mutations. After 2 hours of hybridization at room temperature, spots were washed with 0,3 M $NaNO_3$ 10 mM Phosphate solution (pH 7) to remove nonspecifically bound nanoparticle probes. The spots were treated with Ag nanoparticle suspension to enhance the SERS. After drying of the spots, they were ready for SERS analysis (Figure 2.12).

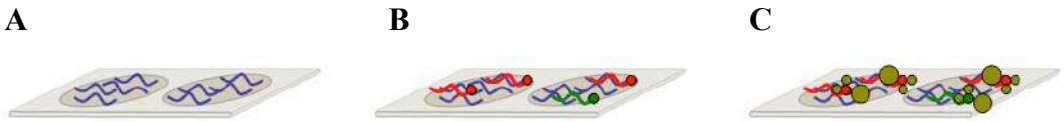


Figure 2.12 Spotting of PCR amplicons on a poly-L-Lysine coated slide (A), hybridization of SERS probes with the immobilized PCR amplicons (B), and staining with colloidal silver nanoparticles before SERS measurement (C).

2.12 SERS Analysis

The spots were analyzed with a completely automated Renishaw InVia Reflex Raman Microscopy System (Renishaw Plc., New Mills, Wotton-under-Edge Gloucestershire, U.K.) equipped with an 830 nm diode and 514 nm argon ion lasers. The laser power is in the range of 0.2 to 6 mW and the 830 nm diode laser used along with either 50x or 20x objective to locate the laser spot on the surfaces. The wavelength of the instrument was automatically calibrated using an internal silicon wafer and the spectrum was centered at 520 cm^{-1}.

2.13 AFM Analysis

The experiments are conducted with a Park SYSTEMS XE 100 Atomic Force Microscopy (**Park Systems Corp.** KANC 4F, Iui-Dong, 906-10 Suwon 443-766, Korea). Imaging was carried out at ambient temperature in non-contact mode with silicium tips with varying resonance frequencies at a linear scanning rate of 0, 5 Hz.

2.14 Transmission Electron Microscopy

High Resolution TEM measurements were performed on JEOL-2100 HRTEM operating at 200kV (LaB_6 filament) and equipped with an Oxford Instruments 6498 EDS system. Carbon support film coated copper TEM grids were used to analyze samples upon dropping very small drops of samples onto them.

3. RESULTS and DISCUSSION

3.1. Optimization of PCR for Amplification of DNA Fragment containing SNP Region

178 bp long fragment, containing 2121(M694V) mutation in the 10th exon of MEFV gene, is amplified to be used in the developed assay. As a first step, to optimize the PCR conditions and reagent amounts that will be used, gradient PCR was carried out between 50 ºC and 60ºC performing 38 cycles consisting of 95°C for 50 seconds (denaturation), 50-60°C for 50 seconds (annealing) and 72°C for 50 seconds (extension). Running PCR products in an agarose gel showed that annealing temperature around 52-54°C gave the best results and nonspecific bands weren't seen in these temperatures. Figure 3.1 shows the agarose gel photo of gradient PCR products.

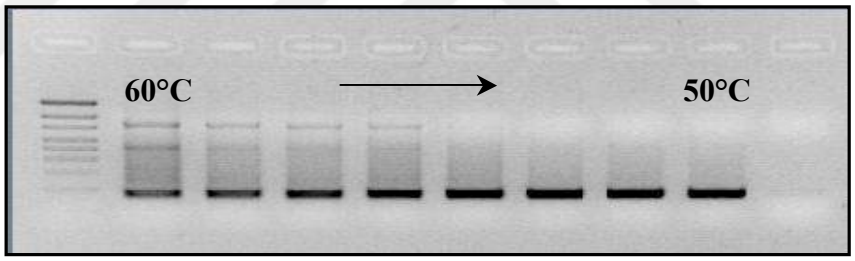


Figure 3.1 Gradient PCR agarose gel photo

Annealing temperature is arranged to be 52°C after gradient PCR and to decrease the smear formation that is a sign of nonspecific binding of taq polymerase, $MgSO_4$ concentration was tried to be optimized. $MgSO_4$ concentration optimization PCR showed that 2 mM $MgSO_4$ concentration is optimum to prevent smear formation. Figure 3.2 shows the agarose gel photo of $MgSO_4$ concentration optimization PCR products.

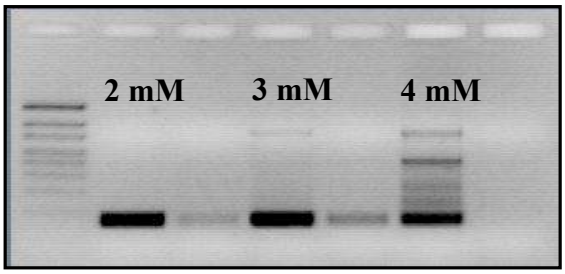


Figure 3.2 $MgSO_4$ concentration optimization agarose gel photo

After this step, PCR optimization is completed and optimum PCR conditions are demonstrated in Table 3.1 and 3.2. An agarose gel photo of PCR products that are amplified according to the optimized conditions is shown in Figure 3.3.

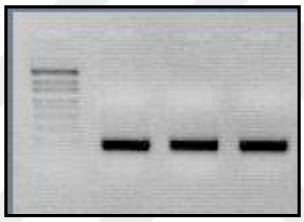


Figure 3.3 Optimum PCR agarose gel photo

Table 3.1 Amounts of PCR reagents in optimum PCR

	Stock Concentration	Optimum Concentration	Amount
Reaction Buffer	10x	1x	5 µl
MgSO4	20mM	**2 mM**	5 µl
dNTP	10mM	400 µM of each	2 µl
Forward Primer	10µM	~0,5 µM	3 µl
Reverse Primer	10µM	~0,5 µM	3 µl
Taq Polymerase	5 u/µl	~1,5 u / 50 µl	3/8 µl
DNA	---		4 µl
dH2O	---		27 µl
Total	---	---	50 µl

Table 3.2 PCR conditions in optimum PCR

	Temperature	Time	Cycle
Denaturation	94°C	50 seconds	38
Annealing	**52°C**	50 seconds	
Elongation	72°C	50 seconds	

3.2. Preparation of SERS Probes

Rhodamine B and TAMRA dyes that have free carboxyl groups are activated to make the dyes become ready for attachment of amine groups of the target molecules. In order to activate dyes, two methods were tried. These methods utilize TSTU and DIPEA in different organic solvents. In this study, the activation of dyes was accomplished effectively using the first method in which acetonitrile was used, and thereafter the first method was chosen for the activation of dyes.

During preparation of SERS probes, a spacer molecule, containing a thiol group that will bind to GNPs, and an amine group that will bind to the activated dyes should be used to combine GNPs and activated dyes. In order to accomplish the attachment of dyes and GNPs; lysine, cysteine, and cysteamine were chosen as spacer molecules because of the fact that they are cheap and abundant according to their expensive and rare counterpart, 6-aminohexanethiol ($HS\text{-}(CH_2)_6\text{-}NH_2$). In addition to 3 alternative spacer molecules, two methods were used to attach the activated dyes to the GNPs.

In the first method, surfaces of GNPs were modified with the spacer molecules, and then the activated dyes were allowed to interact with the free amine group of spacer molecules. In this method, upon modification of GNPs with spacer molecules, they tended to aggregate because of weak molecular interactions such as hydrogen and hydrophobic bonds, which made the sufficient attachment of activated dyes to the spacer molecules more difficult. Therefore, attachment of activated dyes to the GNPs is not possible with this method.

The second method, in which the spacer molecules were attached to the activated dyes prior to the attachment of spacer molecules to the surface of GNPs, gave better results with all 3 spacer molecules. Therefore, the second method was chosen to bind the activated dyes to the surfaces of GNPs.

Lysine, cysteamine, and cysteine molecules, as 3 alternative spacer molecules, are decided to be used for thiolation of activated dyes to attach them to the GNPs. Rhodamine B dye was chosen as a model dye for selection of the most effective spacer molecule that will be used in preparation of SERS probes. Among the spacer molecules used, cysteamine and cysteine were showed to give satisfactory results in binding of activated dyes to the surface of GNPs (Figure 3.4 and 3.5). Although both of these spacer molecules are appropriate for SERS probe preparation, probes prepared with cysteamine were demonstrated to give relatively stronger SERS signal. Therefore, cysteamine was chosen as a spacer molecule for the probe preparation process in the following experiments. Figure 3.4 and 3.5 show the SERS spectra of Rhodamine B probes prepared with cysteine and cysteamine respectively. SERS spectra were obtained after staining with silver colloids to enhance the signals of SERS probes.

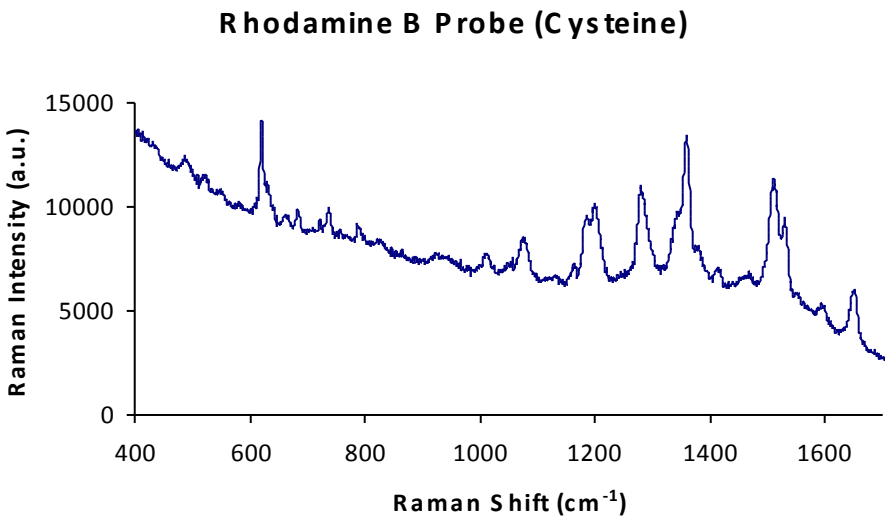


Figure 3.4 SERS spectrum of Rhodamine B attached SERS probes using Cysteine as a spacer molecule

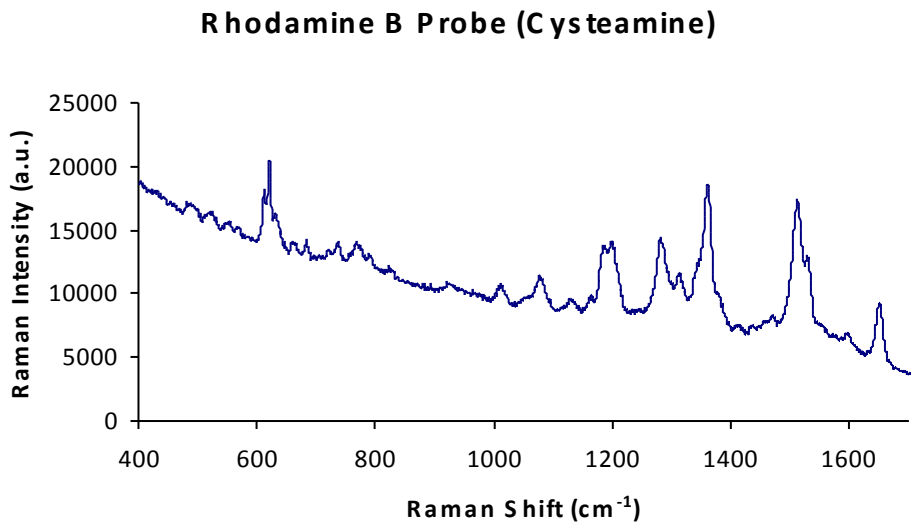


Figure 3.5 SERS spectrum of Rhodamine B attached SERS probes using Cysteamine as a spacer molecule

The SERS probes that will be used in the developed assay were prepared with Rhodamine B and TAMRA dyes. Both dyes were activated using TSTU and DIPEA in acetonitrile solution. After activation of both dyes, they were incubated with spacer molecule cysteamine for 24 hours. Then, the spacer molecule attached dyes were left for incubation in GNP suspension to have control SERS probes. The control SERS probes, containing just activated dyes on their surface, were prepared to be used to prove the attachment of oligonucleotide probes to the surface of actual SNP detection SERS probes by comparing their SERS signal intensities. For preparation of SERS probes that will be used to detect the SNPs, the spacer molecule attached activated dyes and oligonucleotide probes were left together for incubation with GNPs to make them mutually attach to the surface of GNPs. Since both the spacer molecule attached dyes and oligonucleotide probes compete to attach to the surface of GNPs, fewer dyes will attach compared to the probes prepared with just the dyes. Therefore, the intensity of the SERS signal of the oligonucleotide and dye attached probes was expected to be lower than the control probes. Figure 3.6 and 3.7 show the SERS spectra of Rhodamine B and TAMRA probes respectively.

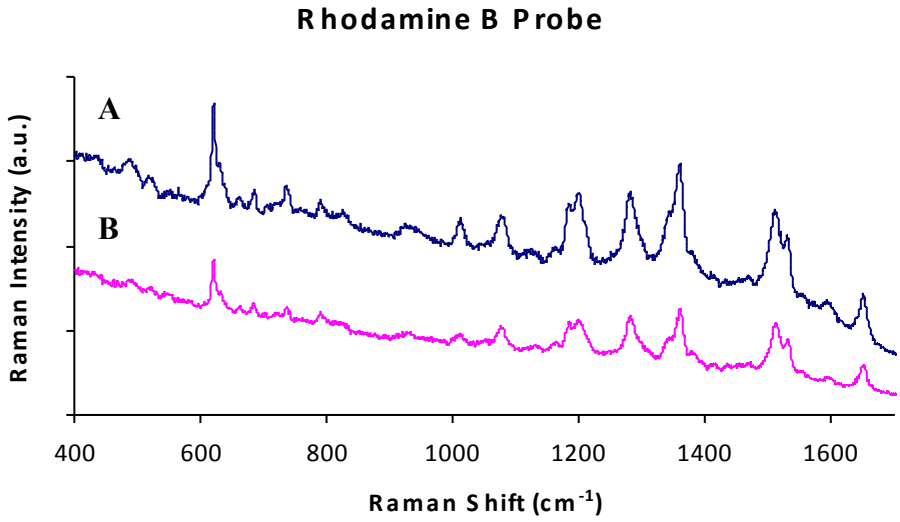


Figure 3.6 The SERS spectrum of Rhodamine Probes without (A) and with (B) oligonucleotide probes

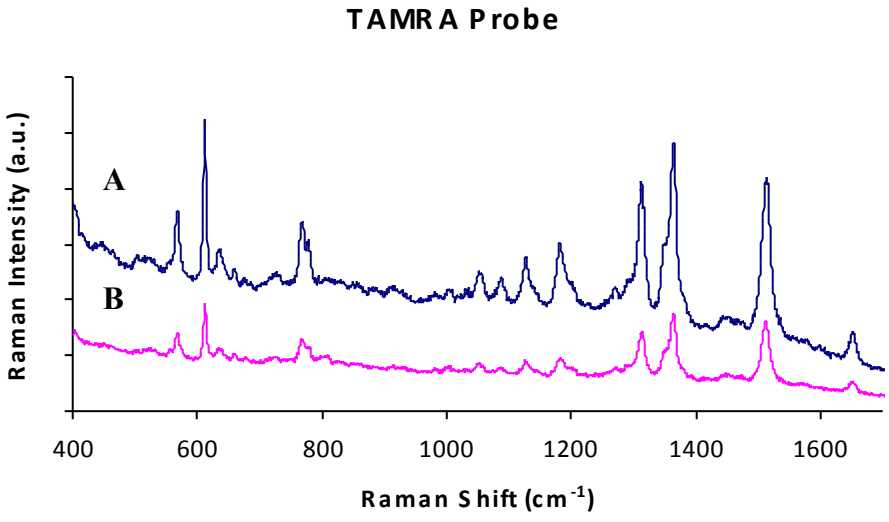


Figure 3.7 The SERS spectrum of TAMRA Probes without (A) and with (B) oligonucleotide probes

During preparation of SERS probes, one of the most significant problems; formation of aggregates and undesired attachment of probes to the walls of ependorf tubes decrease the efficiency of reactions. Therefore, to prevent formation of aggregates and decrease the tendency of probes to attach to the walls of ependorf tubes, SDS, in 0,01% final concentration, was added to the reactions to overcome this problem. SDS is thought to increase the hydrophilicity of probes and prevent formation of aggregates.

3.3. Spotting DNA on Poly-L-Lysine slides

For hybridization process, DNA fragments were needed to be single stranded. Therefore prior to spotting DNA, they were denatured at 95ºC for 5 minutes and then to keep the DNA fragments single stranded, they were put on ice for 1 min. Then, DNA fragments were chemically cross-linked to the poly-L-Lysine glass surface by UV illumination. To have high quality spots, UV energy level, DNA amount, and UV illumination period were optimized.

After DNA solution was dropped and dried, the spots were washed to remove unbound DNA, and then UV illumination was applied in different energy levels ranging from 200 μJ to 400 μJ. The interaction of DNA and poly-L-Lysine slide was analyzed using Atomic Force Microscopy (AFM) and it was found that optimum DNA cross-linking happened between energy levels of 250-350 μJ.

To optimize the DNA amount that is spotted to poly-L-Lysine slides, PCR products having 40 ng/μl (average concentration of PCR products after isolation from agarose gel) concentrations were used. 2 μl of denatured DNA was spotted in every step to have spots consisting of 2, 4, 8, 10, 16 μl of denatured DNA in a single spot and then spots were illuminated with 350 μJ UV to crosslink the denatured DNA covalently to the surface. Analysis with AFM showed that optimum attachment of DNA is seen in the spot consisting of 10 μl of DNA. The spots prepared with 4 and 10 μl of DNA are shown in Figure 3.8 and 3.9 respectively.

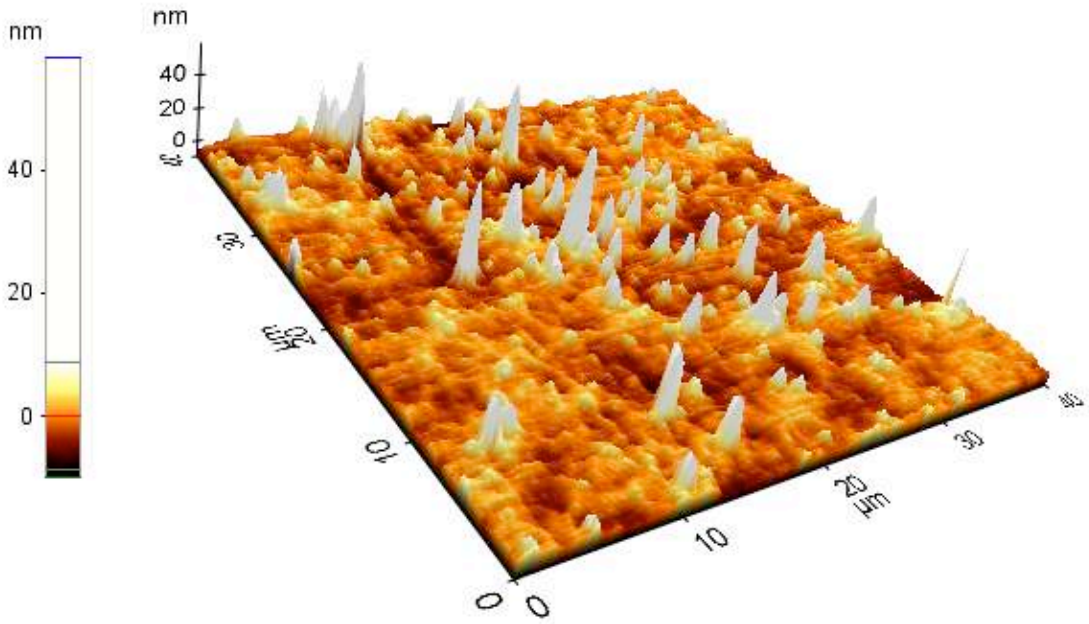


Figure 3.8 The AFM image of the spot that consist of 4 µl of DNA on a poly-L-Lysine glass

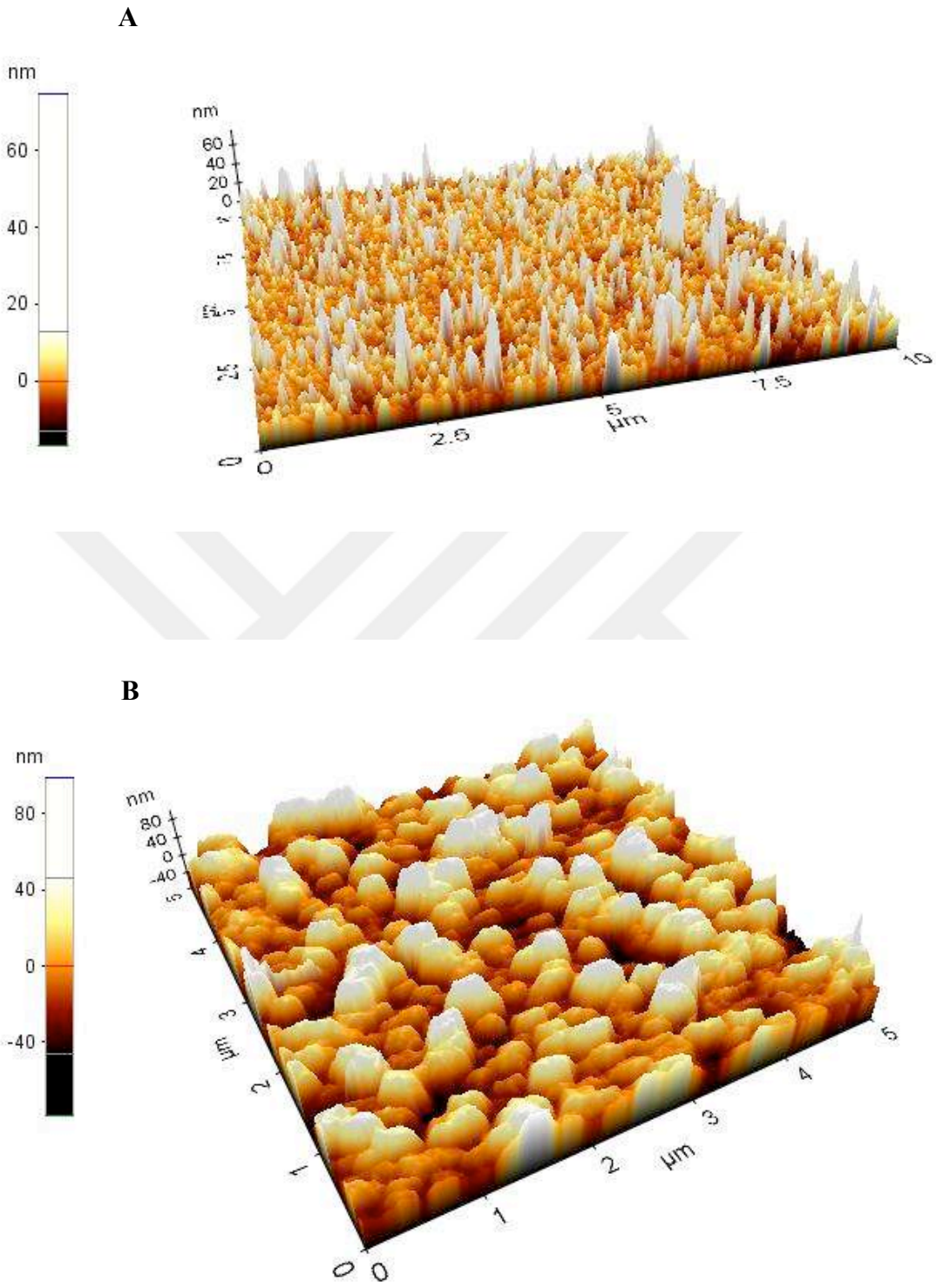


Figure 3.9 The AFM image of the spot that consist of 10 µl of DNA on a poly-L-Lysine glass (A) and a more zoomed image of the same spot (B)

As a last step for optimization of DNA attachment to poly-L-Lysine coated glass slide surface, UV illumination period was optimized. After denaturation of DNA, 2 µl of denatured DNA was dropped in every step to have a spot consisting of 10 µl of denatured DNA (400 ng). To find the optimum way to have high quality spots, UV cross-linking was performed in every step in the first method, and it was performed just once after the last step in the second method. The AFM analysis showed that the spot prepared according to the first method was much better than the one prepared according to the second method. There were few DNA fragments in the spots that were prepared using the second method, with respect to the ones prepared with the first method. Also, DNA fragments of the spots prepared using the first method were evenly distributed on the surface. The AFM image of spots prepared according to the second and first methods are demonstrated in Figure 3.10 and 3.11 respectively.

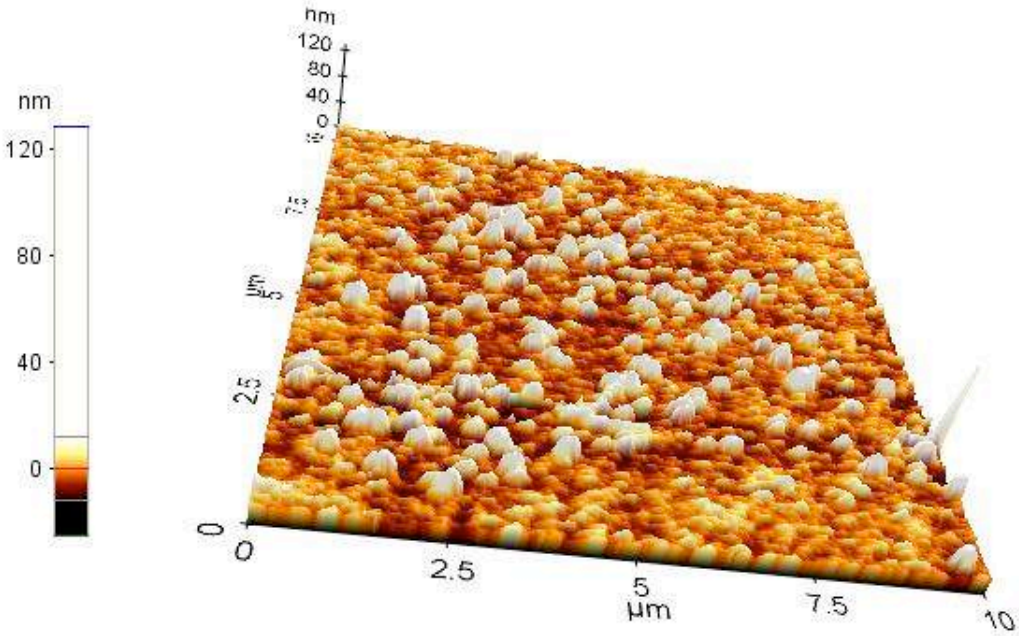


Figure 3.10 The AFM image of the spot that is prepared according to the second method.

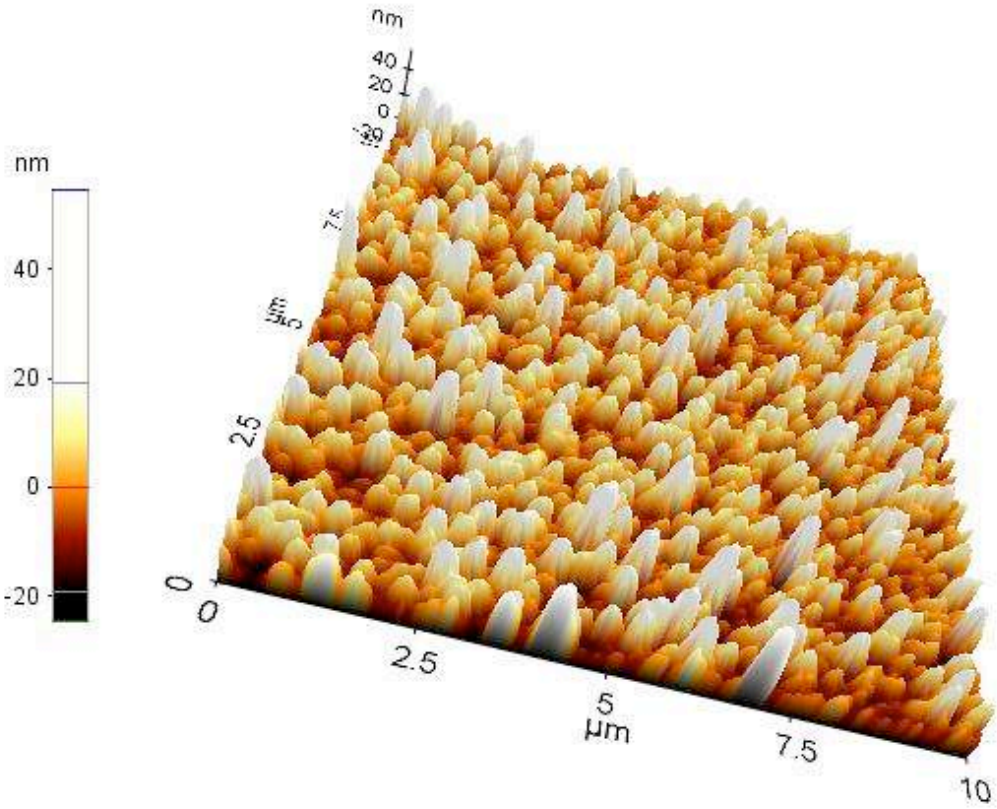


Figure 3.11 The AFM image of the spot that is prepared according to the first method.

3.4. Hybridization of Probes to the Genomic DNA and SERS Analysis

SERS analysis of samples is normally carried out using CaF_2 slides which are not SERS active. Therefore, no background signal is detected in the SERS. In this study, PCR amplified DNA fragments were spotted on poly-L-Lysine coated glass slides and after hybridization of prepared SERS probes to the spotted target DNA, SERS analysis was carried out directly on these glass slides. Thus, determination of background signal by SERS analysis of poly-L-Lysine coated glass slides was significant to justify the results of hybridization. The SERS spectrum of poly-L-Lysine coated glass slide is demonstrated in Figure 3.14.1A.

After determination of background signal, hybridization studies were carried out. A model of the procedure for the detection of the SNP on one spot is demonstrated on Figure 3.12. Each dye chemically attached to GNPs represents the one probe (allele). The SERS probes were prepared using two Raman active dyes; TAMRA and Rhodamine B are used for the detection of the M694V mutation on exon 10 of MEFV gene.

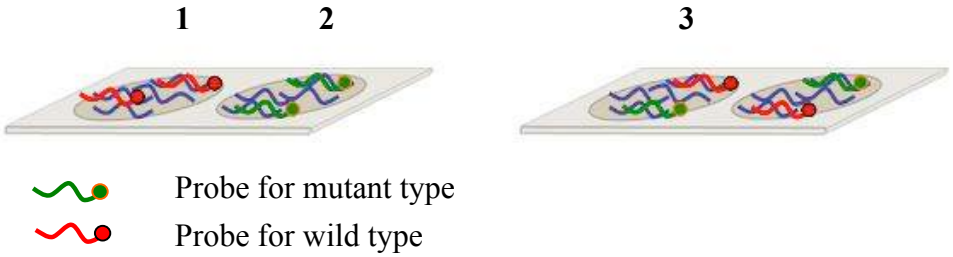


Figure 3.12 Demonstration of hybridization of probes to immobilized PCR products of healthy (1), homozygote (2) and heterozygote (3) patients for M694V mutation.

Upon hybridization of probes with the immobilized PCR products on poly-L-lysine coated surfaces, a colloidal silver nanoparticle staining is necessary for further enhancement of the Raman scattering. This step is necessary due to the poor surface plasmon formation on 13 nm GNPs. It was demonstrated that no SERS signal is detected from a single GNP, modified with activated dyes[48]. This is because, nanoparticles are required to be close enough to each other and the surface plasmons of GNPs are needed to

coincide to enhance Raman scattering. Therefore, to accomplish the enhancement of SERS signal, silver colloidal solution was used to obtain SERS signal from spots hybridized with SERS probes. Figure 3.14.1 illustrates the efficiency of the hybridization and the approach using the probe prepared with TAMRA dye. The SERS signal of the hybridized spot without any further silver staining is shown in Figure 3.14.1 B. The signal resembles the background signal and there is no clear peak coming from the TAMRA dye of the SERS probe. After silver staining of the spot, resolution and the intensity of the spectrum increases (Figure 3.14.1 C).

In order to show the hybridization efficiency from a different approach, light microscopy images of spots after hybridization were also analyzed. 2 spots were prepared with PCR products of a healthy person. Then one of the spots was hybridized with mutant type SERS probes (prepared with Rhodamine B) and the other spot was hybridized with healthy type SERS probes (prepared with Rhodamine B). There was no hybridization of mutant type SERS probes, and healthy type SERS probes were effectively hybridized to the spot. The light microscopy images of both spots after hybridization and stringent wash are demonstrated in Figure 3.13.

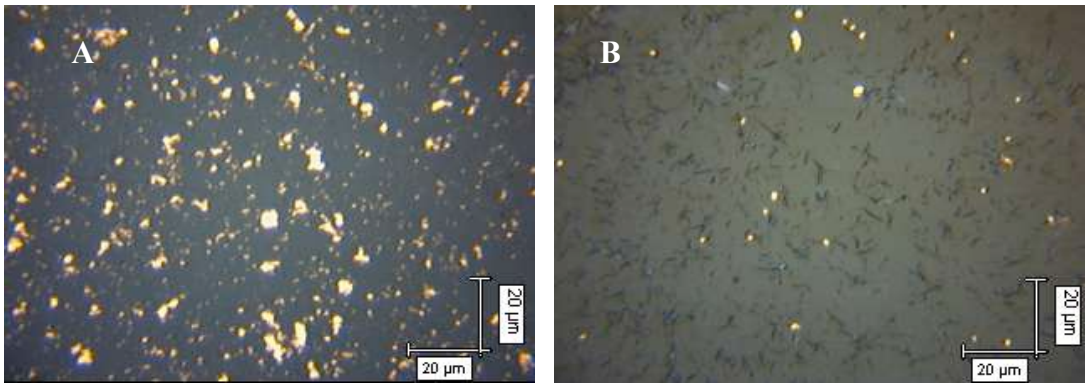


Figure 3.13 Light microscopy images of the spots, prepared with PCR products of healthy person, after hybridization of healthy type SERS probe (A) and mutant type SERS probe (B).

Only two dyes and dependently two probes are satisfactory to screen as many as polymorphisms in the target gene, however, for every SNP in the genome, different oligonucleotides targeting each specific SNP are necessary. One probe for wild type for the specific SNP and one for mutated allele is adequate. If the individual is healthy or mutated, the SERS spectrum from only one dye is observed. If the individual is heterozygous, the two overlapping SERS spectra are observed. The SERS spectra obtained with wild type, heterozygous and mutated samples are demonstrated on Figure 3.14.2. SERS probes for mutant type and healthy type are prepared with TAMRA and Rhodamine B dyes, respectively. When the spot, prepared with the DNA of the patient whose genotype is homozygous mutant, was hybridized with both probes; only the SERS spectrum of TAMRA dye was observed (Figure 3.14.2 A). The spot prepared with healthy person's DNA gave only the SERS spectrum of Rhodamine B (Figure 3.14.2 B). In times of heterozygosity for the mutation, both dyes were hybridized to the spot and the overlapping SERS spectrum of both dyes was observed. 1070 and 1195 bands were obtained from Rhodamine B and 1125 and 1310 bands were obtained from TAMRA in the overlapping spectrum. There are also some bands that are new, lost or weakened in the overlapping spectrum of both dyes. For example, 1180, 1510, and 1645 bands from spectra of both bands are almost lost in the overlapping spectrum. Also, the 1467 band in the overlapping spectrum doesn't seen in the individual probes' spectra (Figure 3.13.2 C). These changes could be from the vibrational changes of the bonds because of the interaction between dye molecules during hybridization.

With the accomplishment of the demonstration of hybridization of both probes to the spot and the overlapping SERS spectrum, it is demonstrated that with an intelligent dye choice, detection of more than one SNP mutation with single Raman measurement on the same spot is feasible.

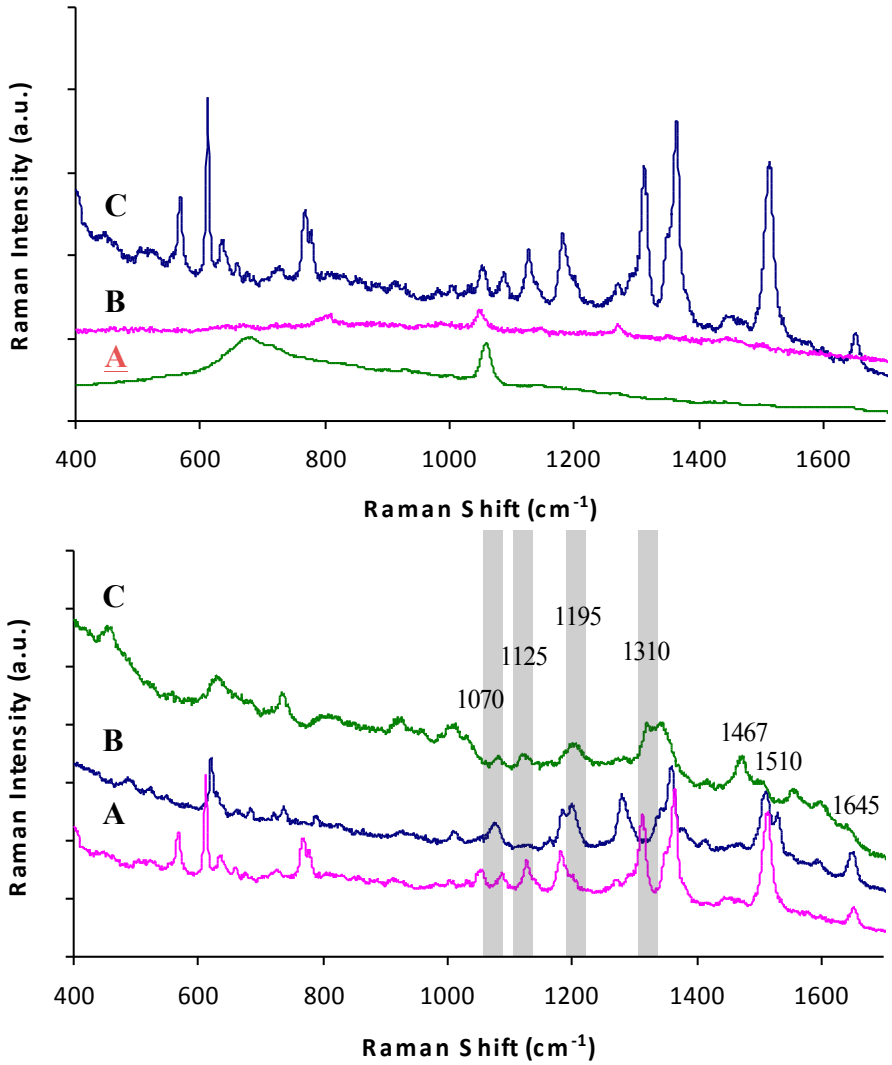


Figure 3.14. 1) SERS spectra of the background after staining with colloidal silver nanoparticles (A), SERS spectrum of the surface after hybridization of the probe with the immobilized PCR amplicons (B), and Demonstration of the hybridization efficiency of the TAMRA labeled SERS probes. SERS spectrum of colloidal silver nanoparticles treated surface after the hybridization with SERS probes (C). **2)** The SERS spectra of the hybridized probes on PCR amplicons on surfaces. TAMRA and Rhodamine B dyes were used for the preparation of the SERS probes. The SERS spectrum of only TAMRA when individual is homozygous mutant (A), the SERS spectrum of only Rhodamine B when individual is homozygous wild-type (B), and The overlapping SERS spectrum of TAMRA and Rhodamine B when the individual is heterozygous mutant (C) are observed. The significant spectral differences are indicated on the spectra with bands.

4. CONCLUSIONS

SNPs, which are the most common polymorphisms throughout the human genome, constitute the 90% of the human DNA polymorphisms. Detection of SNP is performed with utilization of several hybridization techniques using fluorescent or chemiluminescent molecular probes, enzymatic cleavage, sequencing, mass spectroscopy, pyrosequencing, flow cytometry and high-density microarray chips. Although these are standardized methods for determination of polymorphisms, they have some disadvantages such as high cost, being not proper for high-throughput analysis and long processing time. There is still a need to improve the speed and reduce the cost. This can only be achieved with a technique capable of multiplexing. All microarray screening techniques are based on fluorescence detection and they are not suitable for multiplex detection.

M694V is the most common mutation among the FMF patients and M694V/M694V is the most prevalent genotype. M694V SNP mutation is used in this study to develop and test the feasibility of a microchip system to detect SNP mutations in a single spot. In this study it was demonstrated that detection of SNPs on a single spot with SERS is possible. One probe was designed for each allele that may be wild type or mutated. Each SERS spectrum of one of the probes is inferred as homozygosity of genotype. The appearance of the overlapping SERS spectrum originating from both probes indicates the heterozygosity of the genotype. The promising results indicate that it is possible to detect SNPs using this approach. Although it is demonstrated that it is feasible to determine the genotype of a mutation in a single spot and this approach is proper for multiplex detection of SNPs, there are some problems that should be overcome such as non-specific and insufficient hybridization of probes to the spots. Because the interaction between oligonucleotide probes and dyes on the GNPs affects the interaction between oligonucleotide probes and target DNA fragments, it should be noted that the Raman active dyes used in probes should not hinder the oligonucleotide and target DNA hybridization. Besides, electrical charges and the chemistry of Raman active dyes result in the non-specific interaction of SERS probes with the spots. Thus, to reduce the non-specific binding of SERS probes, dye selection should be well considered.

The next step will be the construction of a chip system based on this study. For each SNP points, two probes are enough to detect the possibilities. Preparation of probes with an intelligent dye choice will result in the detection of SERS signals of several probes in a single SERS spectrum. Then, all the genotypic possibilities of more than one SNP (theoretically tens of SNPs) will be detected in a single spot which will cut the cost and time in the detection process.

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