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**THE LOCALIZED SURFACE PLASMON RESONANCE (LSPR) RESPONSE OF
GOLD AND GOLD/SILVER METAL NANOSTRUCTURES TO PROTEIN
BINDING AND GLOBAL REFRACTIVE INDEX CHANGES**

By

Srinivas R. Beeram

A Dissertation
Submitted to the Faculty of the
Graduate School of the University of Louisville
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

Department of Chemistry
University of Louisville
Louisville, Kentucky

August 2010

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A Dissertation Approved on

August 06 2010

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Dedicated to my Dad

Beeram Yadava Reddy

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ABSTRACT

THE LOCALIZED SURFACE PLASMON RESONANCE (LSPR) RESPONSE OF GOLD AND GOLD/SILVER METAL NANOSTRUCTURES TO PROTEIN BINDING AND GLOBAL REFRACTIVE INDEX CHANGES

SRINIVAS R. BEERAM

(AUGUST 06 2010)

This dissertation describes new procedures for synthesizing and purifying Au and Au/Ag nanoplates directly on surfaces as well as controlled binding of proteins to their surface. It also describes fundamental changes in the optical properties of Au and Au/Ag nanoplates upon interaction with different liquids, proteins, or nanoparticles. Finally, this dissertation describes the highly selective and sensitive label-free detection of proteins in an immunoassay format using the optical properties of Au and Au/Ag nanostructures.

A chemical seed-mediated growth procedure allowed the synthesis of Au nanoplates directly on surfaces with an initial 23% yield. Purification by either sonication or adhesive tape led to surfaces coated with ~90% Au nanoplates. Subsequent growth of Ag enabled the synthesis of ~90% Ag-coated Au nanoplates (Au/Ag) directly on the surface. Thiol self-assembly methods allowed select functionalization of the nanoplate terraces or edge sites with human anti-IgG or Au nanoparticles with controlled coverage and binding distance. Characterization of the nanostructures by UV-vis spectroscopy, infrared

spectroscopy, atomic force microscopy (AFM), and scanning electron microscopy (SEM) confirmed the size, shape, composition, and protein or nanoparticle functionalization of the metal nanostructures.

The localized surface plasmon resonance (LSPR) band of Au and Au/Ag nanostructures is sensitive to various liquid environments with a global refractive index (RI) sensitivity that follows as Au/Ag nanoplates (650 - 439 nm/RIU) > Au nanoplates (195 nm/RIU) > Au nanospheres (89 nm/RIU). The local LSPR response to protein binding was 2-3 times larger when proteins were bound to the edge sites of nanostructures compared to terrace sites for similar coverages because the plasmon fields are more intense at these sites. The LSPR response increased as the distance between the protein and the metal decreased in fairly good agreement with theory.

The LSPR band of the metal nanostructures functionalized with human anti-IgG at the edge sites shifts in the presence of human IgG protein due to a change in the nanostructure environment following the highly selective antibody-antigen interaction with a limit of detection of 1 pg/mL. The sensitivity to human IgG followed the order Au/Ag nanoplates $\approx$ Au nanoplates > Au nanospheres. The nanoplates do not respond to goat IgG below a concentration of 1000 ng/mL, showing excellent selectivity. This highly sensitive, selective, label-free, simple, and low cost method for sensing proteins may find use in biomedical applications. The long response times, about 24 hours for low protein concentration, needs improvement.

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CHAPTER I

INTRODUCTION

1.1. Background on metal nanostructures synthesis and their use

Metal nanostructures have physical and chemical properties that are different from their bulk. Nanomaterials are the candidates of interest in biosensing because of their small size, large surface-to-volume ratio, and size and shape dependent optical properties. Any binding event of an analyte to the receptor bound to the nanoparticle causes the optical properties of nanoparticles to change due to a change in the local refractive index around the nanoparticles environment which is not observed in their bulk counterpart. Nanomaterials can be used in various applications like sensing, catalysis, drug delivery, tissue engineering and tumor destruction. Au metal nanoplates are the ideal metal nanostructures for our work for determining the effect of binding location of proteins on the shift in λ_{max} of the localized surface plasmon resonance (LSPR) spectrum because they 1) are atomically smooth, which allows easy visualization of molecules attached to the surface by AFM and 2) have different sites with different LSPR properties.

The following section focuses on the synthesis of metal nanoplates and their various applications. Several methods exist in the literature for synthesizing Au and Ag metal nanoplates and can be categorized into synthesis in solution and synthesis directly on surfaces. Solution-based methods for synthesizing Au nanoplates include chemical, electrochemical,¹ and photochemical^{2, 3} reduction of an Au complex (usually AuCl_4^-) in

the presence of a stabilizer. Some common chemical reducing agents include ethylene glycol,⁴⁻⁶ a polyamine,^{7, 8} lemongrass extract of a plant,⁹ polyvinylpyrrolidone (PVP),¹⁰ ascorbic acid,¹¹ tartaric acid,² sodium citrate,¹²⁻¹⁴ salicylic acid,¹⁵ natural humic substance,¹⁶ and formaldehyde¹⁷ in the presence of stabilizers, such as cetyltrimethylammonium bromide (CTAB), cetyltrimethylammonium chloride (CTACl), PVP, or ionic liquids. The chemical reduction often involves heating,^{2, 4-6, 8, 10, 12-16} and in some cases, the reducing agent and stabilizer are the same.^{8, 10, 16, 18} For example, Huang and co-workers synthesized Au nanoplates of various sizes by heating an aqueous solution containing Au salt, cetyltrimethylammonium bromide (CTAB) and trisodium citrate by varying the composition of growth solution.¹³ Xia reported the use of PVP for synthesizing micron-sized Au nanoplates in solution by a thermal approach, controlling the molar ratio of PVP and HAuCl₄.¹⁰ Au nanoplates have also been synthesized within PVA polymer films¹⁸ where PVA is also the stabilizer, in ionic liquids¹⁹ with heating, and in liquid crystals made of block copolymers²⁰ at room temperature. Photochemical methods used CTACl and PVA as stabilizing agents,^{2, 3} and in one case laser ablation of solid Au metal in solution led to Au nanoplates.²¹ Solution-based chemical methods for synthesizing Ag metal nanoplates involve the laser ablation of Ag metal,²¹ or chemical reduction of Ag⁺ by PVP^{10, 22} and dimethylformamide²³ through heating in the presence of a stabilizer. Light²⁴⁻²⁸ and heat²⁹ induced conversion of nanospheres to nanoplates has also been reported. Mirkin and co-workers synthesized Ag nanoprisms of different thickness in solution by varying the concentration of sodium borohydride during a thermal conversion of spheres to nanoplates²⁹ and by light induced reduction to synthesize Ag nanoplates in solution.²⁶ Bastys *et al* synthesized high aspect ratio Ag

nanoprisms by illumination with either light-emitting diodes (LEDs) or white light combined with different color filters.²⁴ Other methods involve heat³⁰- and light³¹⁻³³-induced shape changes of nanoplates. Lombardi and co-workers synthesized hexagonal Ag nanoplates from triangular nanoplates in solution by irradiating with a sodium lamp,³¹ while Xu and co-workers used sodium lamp illumination to synthesize Ag nanoplates in solution, converting them into nanodisks and then regenerating them back to nanoplates by increasing the citrate concentration along with further illumination.³²

Several groups have reported the chemical seed-mediated synthesis of Au and Ag nanoplates in solutions.³⁴⁻⁴⁰ Mirkin and co-workers synthesized Au nanoprisms in solution with uniform edge lengths and thickness using a seed-mediated approach involving solutions containing a capping agent, Au salt, reducing agent and NaOH.³⁹ Ha *et al.* reported the synthesis of Au nanoprisms by a seed-mediated approach, showing that the presence of iodide in the growth solution is responsible for nanoprism formation.³⁷ The concentration of iodide in the growth solution is also important for the formation of either triangular or circular plates. Carroll and co-workers synthesized truncated triangular Ag nanoplates in solution using a seed-mediated method in the presence of CTAB under alkaline conditions.³⁵ High yields of Ag nanoplates (>95 %)³⁴ and Au nanoplates (96 %)⁶ in solution have been reported both with³⁹ and without^{6, 34} purification. The main disadvantage of nanoplates synthesized in solution is that they need to be later assembled on a surface for various sensor or other plasmonic-based applications. The large excess of surfactant or polymer usually present can often make the assembly of well-isolated nanoplates on surfaces a challenge. Han and co-workers recently assembled Ag nanoprisms by two different methods on a surface from solution

and observed their different optical properties,⁴¹ but this has not yet been reported for Au nanoplates.

There are examples of synthesizing Au and Ag nanoplates directly on a surface.⁴²⁻⁴⁸ Van Duyne and co-workers pioneered the use of nanosphere lithography (NSL), a method to fabricate well-ordered arrays of Ag, Cu, and Al nanoplates directly on surfaces by vapor deposition of the metal of interest through an ordered array of polymeric spheres acting as a mask.⁴⁸⁻⁵⁰ Our group and others reported on the use of seed-mediated growth of Au or Ag nanoplates directly on surfaces.^{42, 43, 45-47} Seed-mediated synthesis of metal nanostructures is a simple benchtop wet chemical method that involves the synthesis of metal nanoparticle seeds that are attached chemically onto the surface through covalent linkage and growth of these nanoparticle seeds into nanoplates by the reduction of metal salt in the presence of a stabilizer (CTAB or PVP). The source of the CTAB we used to grow nanoplates directly on the surface was from Aldrich (95 %) which was critical in forming nanoplates.⁵¹ This method is beneficial over NSL in that it is an all chemical benchtop approach that does not require as many steps or high vacuum metal evaporation systems. Although NSL leads to highly uniform structures on the surface, the nanoplates synthesized by our method are well-isolated from one another and highly crystalline. Geddes and co-workers reported a 80% yield for Ag nanoplates⁴² but not well defined shapes and others reported a 30–60% yield for Au nanoplates.^{45, 46} Sun *et al.* reported a high yield, size-controlled synthesis of Ag nanoplates by galvanic exchange directly on an n-type semiconductor substrate.⁴⁴ We synthesized Au nanoplates directly on surfaces by seed-mediated approach using two different methods. The percent yields of the nanoplates were 23 % and 44 % using two different growth

solutions (GS1 and GS2). We purified the nanoplates samples by using an adhesive tape and ultrasonication to remove the spherical particles that resulted in samples with a % yield of > 90 %. Our purification strategy leads to a higher yield of nanoplates on the surface when compared to other seed-mediated growth methods directly on surfaces. While it may be possible to synthesize Au nanoplates in solution in high yield and assemble them onto surfaces, we have not found any literature examples and believe our method will be better for assembling well-isolated nanoplates on surfaces with no aggregation. Nanoplates have been utilized in various applications, including catalysis,⁵²⁻⁵⁴ drug screening,⁵⁵ controlled nucleic acid release,⁵⁶ surface-enhanced Raman spectroscopy (SERS),¹⁶ and LSPR-based biosensing.⁵⁷ Table 1.1 shows a summary of various reducing agents and stabilizers for nanoplates synthesis in solution and on surfaces.

Au nanoplates are the ideal metal nanostructures for our work for determining the effect of binding location on the shift in λ_{max} of the LSPR spectrum because they 1) are atomically smooth, which allows easy visualization of molecules attached to the surface by AFM and 2) have different sites with different LSPR properties. Our group demonstrated imaging of the location of human anti-IgG bound directly on Au nanoplates as described in chapter III and others have shown by theoretical calculations⁵⁸ and electron energy loss spectroscopy (EELS)⁵⁹ that the electromagnetic field strength of the localized surface plasmons increases in the order of vertex sites > edge sites > terrace sites of metal nanoplates. The energy of the surface plasmons depends on the exact size and shape of the nanoplate, but generally follows the order vertex < edge < terrace.⁵⁹ There have been some qualitative studies showing location-dependent shifts in λ_{max} upon

Table 1.1. Summary of various reducing agents and stabilizers used in the synthesis of gold and silver metal nanoplates in solution and on surfaces.

Nanoplates in solution	Reducing agent	Stabilizer	Seed	Ref
Gold	Sodium citrate	PVP	No	12
Gold	Orthophenylenediamine	Orthophenylenediamine	No	7
Gold	Ascorbic acid	PVP	No	11
Gold	Light	PVA	No	3
Gold	Polyethyleneimine	Polyethyleneimine	No	8
Gold	Humic substance +heat	Humic substance	No	16
Gold	Ethylene glycol +heat	PVP	No	4, 5, 6
Gold	Salicylic acid + heat	PDDA	No	14
Gold	Sodium citrate + heat	CTAB	No	13
Gold	PVP	PVP	No	10
Gold	PVA	PVA	No	18
Gold	Tartaric acid	CTACl	No	2
Gold	Light	CTACl	No	2
Gold	Formaldehyde	Ionic liquid	No	17
Gold	Aniline	Polyaniline	Yes	36
Gold	Ascorbic acid	CTAB	Yes	39
Gold	Electrochemical	PVP	No	1
Gold	Ionic liquid + heat	Ionic liquid	No	19
Silver	Ascorbic acid	CTAB	Yes	35
Silver	Ascorbic acid	PSSS	Yes	34
Silver	Ascorbic acid + citric acid	NaAOT	Yes	38

Silver	DMF	PVP	No	23
Silver	PVP + heat	PVP	No	10
Nanoplates on surface	Reducing agent	Stabilizer	Seed	Ref
Gold	PVP	PVP	Yes	45
Gold	Potential + ascorbic acid	CTAB	Yes	43
Gold	Ascorbic acid	CTAB + PVP	Yes	46
Gold	Ascorbic acid	CTAB	Yes	47
Silver	Ascorbic acid	CTAB	Yes	42

analyte binding to metal nanostructures,^{57, 60} but none directly correlating the location of the bound analyte with the LSPR spectrum of the same sample on nanoplates or other structures.

1.2. SPR and LSPR

1.2.1. Surface Plasmon Resonance (SPR)

SPR occurs when light is reflected off a thin metal film (usually Au) deposited on quartz when the angle of incidence is greater than the angle of Total Internal Reflection (TIR).⁶¹ SPR can be used for biodetection by observing the change in the SPR resonance angle when a biomolecule binds to the Au surface.⁶¹ For example, Lofas *et al.* utilized SPR to sense immunoglobulin antibodies by observing the critical angle changes when an antibody was bound to the gold film.⁶² Others have used SPR for detection of cell-ligand interactions, DNA, proteins, and nicotine gas.⁶³⁻⁶⁶

1.2.2. Localized Surface Plasmon Resonance (LSPR)

LSPR occurs when the frequency of incident photons matches the collective oscillations of the conduction band electrons of noble metal nanoparticles. Metal nanoparticles like gold and silver have been used for LSPR sensing because of their unique optical properties within the UV-VIS region. The difference between SPR and LSPR is that in SPR the surface plasmon is propagating on a thin metal film where as in LSPR the plasmon is localized on the surface of the metal nanoparticle. Table 1.2 shows a comparison of SPR and LSPR.

Table 1.2. Comparison between SPR and LSPR.

Characteristic	Surface Plasmon Resonance (SPR)	Localized Surface Plasmon Resonance (LSPR)
Modes	Angle shift and wavelength shift	Wavelength shifts and extinction changes
Substrate	Thin metal films	Metal nanoparticles
Propagating Distance	10-100 μm along x-y direction	Plasmon is localized
Label-free detection	Yes	Yes
Commercial availability	Yes	No
Multiplexing	Yes	Yes
Small molecule sensitivities	Good	Better
Cost	US\$ 150,000-300,000	US\$ 5000
Spatial resolution	$\sim 10 \times 10 \mu\text{m}$	1 nanoparticle

1.3. Background on LSPR

When the frequency of incident photons matches the collective oscillations of the conduction band electrons of noble metal nanoparticles, localized surface plasmon resonance (LSPR) occurs⁶⁷⁻⁷¹ which results in a strong absorption band or multiple bands in the visible region for metals such as Au and Ag. The intensity and wavelength of the LSPR band depends on several factors, including the composition, size, and shape of the nanoparticles as well as the dielectric properties of the environment surrounding the metal and their local proximity to other metal nanoparticles.⁶⁷⁻⁷¹ Metal nanostructures can be used in sensing by three different ways based on their visible optical properties. The first involves monitoring shifts in the LSPR band due to nanoparticle aggregation or changes in the nanoparticle-nanoparticle distance. This is usually performed with nanoparticles in solution,⁷²⁻⁷⁵ but can be achieved in films.^{76, 77} For example, researchers have detected polynucleotides,⁷² proteins,^{73, 75} solvents,⁷⁷ and metal ions^{74, 76} through this scheme. Structural changes in DNA or other dynamic biophysical processes have also been probed by monitoring small changes in nanoparticle-nanoparticle distances optically, which is termed “molecular rulers”.⁷⁸⁻⁸⁰ A second approach involves using the nanoparticles as optical tags^{81, 82} similar to fluorophores or other labels in an immunoassay or for cell imaging.^{13, 83} In the presence of a cell or biomolecule of interest, a metal nanoparticle binds to a surface or the cell and produces a signal that can be measured with a detector or directly imaged microscopically. Ag^{13, 81-83} and Au^{82, 84} nanoparticles and striped metal nanorod biobarcode⁸⁵ are examples. The third approach, which is the focus of our research, involves monitoring the shift in the LSPR band for metal nanoparticles based on changes in the local dielectric environment upon binding of

analyte to the nanoparticle surface. This has recently been termed LSPR spectroscopy^{67, 71} and compared to surface plasmon resonance (SPR) spectroscopy,⁸⁶ which is a well-established technique for monitoring biochemical binding events and reactions at surfaces.

LSPR spectroscopy experiments can be performed on metal nanoparticles dissolved in solution,⁸⁷⁻⁹² thin evaporated metal films,⁹³⁻⁹⁸ or chemically-synthesized metal nanoparticles immobilized on a surface⁹⁹⁻¹⁰⁴ and the optical properties monitored in transmission^{96, 97, 101, 102} or scattering/reflection mode on a collection of nanoparticles¹⁰² or individual nanoparticles.^{105, 106} Figure 1.1 shows the modes of performing LSPR based sensing. Sensing has been performed by detecting changes in the wavelength ($\Delta\lambda$) or intensity of the LSPR band of the metal nanoparticles upon binding of analyte as shown in the cartoon in Figure 1.2. In most cases, the LSPR response exhibits a red shift and increases intensity upon analyte binding due to dielectric changes in the environment.⁶⁷⁻⁷¹

1.4. Refractive Index Sensitivity

There are two ways of performing LSPR experiments: 1) monitoring the response to global refractive index (RI) changes and 2) measuring the response to local binding of analyte.

Bulk RI sensitivity

For RI changes, the sensitivity mainly depends on the bulk LSPR band position. As the wavelength increases, the sensitivity increases. Predictions generally show that the RI sensitivity increases as the bulk LSPR band increases to higher wavelengths and

Figure 1.1. A) LSPR experiments of particles in solution. B) LSPR experiments using surface attached particles. C) LSPR experiments using a single nanoparticle.

Figure 1.2. Cartoon showing general LSPR sensing mechanism. A) LSPR spectrum of a surface attached Au spherical nanoparticle. B) LSPR spectra after attaching the receptor. C) LSPR spectral response after binding analyte to the receptor.

aspect ratio of the metal increases. This is not always the case, however, depending on the extent of radiative damping and the effect of the substrate dielectric for assembled nanostructures.¹⁰⁷ Studies have shown that the bulk refractive index (RI) sensitivity order follows as rods > triangles > spheres.^{108, 109} Chen *et al.* experimentally showed that the RI sensitivity of Au nanostructures followed as branches (703) > bipyramids (540) > rods (288) > cubes (83) > spheres (44).¹¹⁰ RI sensitivity is generally calculated from the slope of the wavelength changes per refractive index unit change. The figure of merit (FOM) followed the order bipyramids (4.5) > rods (1.7) > cubes (1.5) > branches (0.8) > cubes (0.6). The FOM is calculated by dividing the RI sensitivity by the full width at half maximum of the extinction peak of the nanoparticles.

Local Binding

Response to local binding refers to any molecular or biomolecular binding on the surface of the metal nanoparticle. The sensitivity to local binding of molecules decreases exponentially with increasing distance within 40-50 nm from the nanostructure and increases with increasing analyte volume. The global RI sensitivity serves as an upper limit to the local response. It is also predicted that the sensitivity depends on the molecule binding location on the metal nanostructure.

1.5. LSPR sensing of various analytes.

LSPR sensing reports of biological molecules, such as DNA^{60, 80, 81} and proteins,^{67, 97, 102, 103, 105, 111-116} have increased tremendously over the past few years because the method is highly sensitive, simple, low cost, and label-free. Several examples of LSPR spectroscopy for biosensing exist in the literature. Englebienne and co-workers were the

first to report antibody detection by measuring the extinction changes caused by local refractive index changes around Au nanoparticles in solution.⁸⁸ Other solution examples include measurements of biopolymer adsorption kinetics,⁸⁷ ligand-protein interactions,⁹⁰ high-throughput screening of proteins,⁹¹ pH,⁸⁹ and ascorbic acid.⁸⁹ Recently, Yu and Irudayaraj used antibody-functionalized Au nanorods of varying aspect ratio for multiplex biosensing of various IgG.⁹² Most of the solution examples detect proteins in the μM to nM range.

There are a large number of studies using films of metal nanostructures, either evaporated or chemically-synthesized, for LSPR sensing. Chilkoti and co-workers synthesized and assembled Au nanoparticles on the surface of glass^{101, 102} finding that the optimal nanoparticle size was about 39 nm in diameter¹⁰² for streptavidin sensing. Chilkoti also synthesized nanorods and assembled on a glass surface for sensing of streptavidin¹¹⁴ and found that nanorods were more sensitive when compared to that of nanoparticles.¹¹⁴ Rubinstein and coworkers evaporated discontinuous Au films for sensing of avidin and antibodies specific to IgG and hCG antigens.^{94, 97} They detected porphyrins⁹⁸ and vapor molecules⁹⁶ when the Au films were coated with silica or polymers, respectively. Other examples include films of Au nanodot patterns,¹¹⁷ Ag dot arrays,¹¹⁸ Au evaporated onto porous anodic alumina,¹¹⁹ electrodeposited Au,¹²⁰ fabricated silica/Au core/shell particles,¹²¹ Au nanoparticles grown from Au seeds,¹⁰³ Au evaporated on polystyrene,^{122, 123} and Ag films sputtered⁹³ or formed by glancing angle deposition⁹⁵ for the detection of various analytes, such as IgG,⁹⁵ streptavidin,⁹³ aptamer-protein interactions,¹¹⁹ melittin,¹²¹ and BSA.¹⁰³ Chemically-synthesized Au nanoparticles,^{99, 100, 104, 122} shells,¹²⁴ bipyramids,¹²⁵ or nanorods^{115, 126} assembled on

various surfaces, including smooth Au films,¹²² SiO₂,¹⁰⁰ glass,^{104, 125, 126} and an optical fiber⁹⁹ led to optical sensing of avidin,¹⁰⁰ streptavidin,^{99, 126} peptides,¹²² IgG,¹¹⁵ and cholera toxin.¹⁰⁴ Van Duyne and co-workers developed the use of nanosphere lithography^{86, 107, 111, 112, 127-133} for LSPR sensing of streptavidin,¹²⁸ a biomarker for Alzheimer's disease,^{111, 112} and a carbohydrate.⁸⁶ Out of the methods described above, Au nanorods¹¹⁴ and the optical fiber method⁹⁹ displayed detection limits around 100 pM for streptavidin while the Ag nanosphere lithography approach¹²⁸ detected streptavidin below 1 pM.

There are some examples of sensing studies on individual metal nanostructures. Most of these involve measuring the sensitivity to refractive index changes in the environment or a measurement of the sensing figure of merit (FOM) as a function of the metal composition, size, and shape. Examples include Au^{60, 106, 134, 135} and Ag^{108, 136, 137} nanoparticles, Au nanorods,^{105, 138} Au₂S/Au nanoshells,¹³⁹ Ag cubes,^{140, 141} Ag triangular prisms,^{116, 137, 142, 143} star-shaped Au particles,¹⁴⁴ and Au cubic frames.¹⁴⁵ Individual nanoparticles are optically sensitive to adsorbed alkanethiols,^{108, 116, 139} chemical reactions,¹³⁴ and atomic force microscopy imaging.¹⁴³ Examples of biosensing with individual nanoparticles include detection of streptavidin with an Au nanoparticle¹⁰⁶ and Au nanorod,^{105, 138} cytokines with an Ag nanoparticle,¹³⁶ and DNA with an Au nanoparticle coupled to a larger Au nanoparticle.⁶⁰ One of the major benefits of single nanoparticle biosensing is the narrow band width in the spectrum due to the removal of broadening that occurs when measuring the average optical signal from many different particles. This improves the discrimination between small wavelength changes and the sensitivity.¹⁴¹ In addition, single-particle sensing involves smaller volumes and the

Table 1.3. Summary of work performed on various nanostructures along with the detection limits of various analytes.

Nanostructure	Analyte	Detection limit	Reference
Gold nanoparticles on silicon substrate	Avidin	1 $\mu\text{g/mL}$	100
Gold island films	Avidin	50 $\mu\text{g/mL}$	97
Gold nanoparticles	Bovine serum albumin(BSA)	0.01 μM	103
Gold films	Human chorionic gonadotropin β (HCG) antibodies	100 $\mu\text{g/mL}$	94
Silica core gold shell	Mellitin	10 ng/mL	121
Gold nanorods	Anti-rabbit IgG	10 nM	115
Silver films	Anti-rabbit IgG	13 nM	95
Gold films	Anti-rabbit IgG	1 μM	94
Gold films	Anti-mouse IgG	1 μM	94
Gold nanorod multiplexing	IgG	200 nM	92

Silver triangles	Anti-ADDL	50 nM	112
Gold nanoparticles	IgG	0.1 ng/mL	47
Gold nanoplates	IgG	0.01 ng/mL	173
Gold colloids on fiber	Streptavidin	9.8×10^{-11} M (~ 98 pM)	99
Gold nanorods	Streptavidin	0.005 μ g/mL in PBS (95 pM) & 1 μ g/mL in serum (19 nM)	114
Gold nanoparticles	Streptavidin	0.05 μ g/mL	102
Gold nanoparticles	Streptavidin	16 nM	101
Silver sputtered films	Streptavidin	16 nM	93
Silver triangles	Streptavidin	1 pM	172
Single Au nanoparticle	Streptavidin	50 μ g/mL	106
Single Au nanorod	Streptavidin	1 nM	105

potential for massive multiplexing in an array format. Table 1.3 summarizes the list of various nanostructures used in LSPR sensing along with the detection limits for various analytes.

There are several factors that control the LSPR response of metal nanostructures to dielectric changes in the environment, including the size,¹¹⁰ shape,¹¹⁰ and composition^{146, 147} of the metal nanostructure.^{148, 149} The proximity of a nanoparticle to another,^{60, 150} interaction with the substrate,^{107, 122, 141, 151} surface roughness,¹⁵² electric field decay length (or sensing volume),^{67, 107, 114, 127, 132, 138} and volume of the analyte¹³⁸ are also important. The width of the LSPR band,^{71, 141} the minimum detectable wavelength shift, and instrument noise are additional factors.¹³⁸ In general, as the bulk LSPR band of the metal and the aspect ratio of the nanostructure increases, the sensitivity increases since the plasmon decay length increases.^{109, 134, 148} It was also shown that shells are more sensitive than similar diameter filled spheres^{113, 124, 139} and rod-shaped Au nanorattles are more sensitive than Au rods or spheres.¹⁵³ Cubes are less sensitive to refractive index (RI) changes compared to triangles, but have a larger FOM due to narrow peaks.¹⁴¹ Recently, Hafner and co-workers reported a refractive index sensitivity and FOM for star-shaped Au nanoparticles larger than previously reported for other shapes.¹⁴⁴ Coupled nanoparticle pairs are more sensitive compared to individual particles¹⁵⁰ and the sensitivity of metal nanostructures to the environment drops exponentially with distance from the nanoparticle.¹³² In addition, the LSPR response is sensitive to the molecular adsorbate's electronic structure in relation to the LSPR band of the metal nanostructure.⁷¹

The sensitivity to global refractive index changes in the medium represents the upper limit of the possible response to local molecular adsorbates or biomolecules

binding to the metal nanostructures. The LSPR response to these molecular or biomolecular events depends on the fraction of the sensing volume filled by the molecules attached. The sensing volume usually extends 25-50 nm from the nanoparticle surface. The areas within the sensing volume are not equal, though, in terms of their effect on the LSPR band. As mentioned earlier, the sensitivity to the environments drops exponentially with distance, but it is also predicted that the sensitivity to local dielectric changes in the environment (analyte binding) depends on the location of the binding site on the nanostructure. Van Duyne and coworkers predicted that sharp parts of a nanostructure will act as “hot spots”, being very sensitive to local dielectric changes, while flat terrace regions will be comparably less sensitive, or act as “cold spots”.¹⁰⁷ Calculations showed that an Ag nanotriangle could be less than 10% covered, but still exhibit almost its maximum shift if the adsorbate is confined to the edges.¹⁰⁷ There are few experimental examples that support the theoretical prediction. First, Van Duyne and coworkers showed large shifts in the LSPR band for triangular Ag upon non-specific adsorption of antibody onto the Cr adhesion layer, which localized the antibody near the edges (“hot spots”).¹¹² Second, there were very different changes in the optical properties of an individual large Au nanoparticle upon binding of smaller Au nanoparticles through DNA hybridization.⁶⁰ One reason suggested was that the optical shift depends on the binding location.

There are several factors that control the sensitivity of metal nanostructures to a binding analyte. The following equation describes the change in the wavelength of maximum absorbance ($\Delta\lambda_{\text{max}}$) upon molecular binding to the nanostructure:¹⁵⁴

$$\Delta\lambda_{\max} = (S(r)/V_s)[\Delta RI \cdot N \cdot V_A] \quad (1)$$

where V_s is the sensing volume, ΔRI is the difference in the refractive index (RI) between the binding molecule and the medium, N is the number of bound molecules, and V_A is the volume of one individual analyte molecule. $S(r)$ is the spatially-dependent RI sensitivity of the metal nanostructure.¹⁵⁴ The second term, $\Delta RI \cdot N \cdot V_A$, represents the optical mass increase as a result of the bound analyte.¹⁵⁴ This term only depends on the properties of the analyte and the medium; not the metal nanostructure. There is not much one can do to alter variables in the second term, other than to monitor the LSPR response in air before and after analyte binding instead of in aqueous solutions or high dielectric solvents. This is especially true for biosensing applications, since ΔRI is smaller between water and biomolecules compared to that between air and biomolecules. The first term in equation 1, $S(r)/V_s$, depends on the metal nanostructure size and shape and the distance (r) between the bound molecule and the nanostructure surface. V_s is commonly defined as the volume surrounding the nanostructure that contains 95% of its sensitivity.¹⁵⁴ $S(r)$ and V_s can be determined experimentally as a function of different nanoparticle sizes and shapes. $\Delta\lambda_{\max}$ decreases exponentially as r increases. This is due to the exponential decrease in the electromagnetic field enhancement as a function of distance from the nanoparticle surface, known as the electric field enhancement decay length, l_d . The result is that the LSPR band is not sensitive to analyte molecules greater than ~40-50 nm from the nanostructure surface.¹⁰² Mathematical expressions for $S(r)$ for a given nanostructure size and shape involve r , l_d , and S_0 , where r and l_d are as described previously and S_0 is

the bulk sensitivity to global changes in RI. S_0 has recently been found to be independent of the nanostructure geometry for a given metal; it only depends on the initial $\lambda_{\max}$.^{70, 149, 155} Chilkoti and co-workers recently developed an expression for $S(r)$ in their study of Au nanorods as follows:¹⁵⁴

$$S(r) = 3 * S_0 * \exp(-2r/l_d) \quad (2)$$

Substitution into equation (1), gives:

$$\Delta\lambda_{\max} = [3 * S_0 * \exp(-2r/l_d)/V_s][\Delta RI * N * V_A] \quad (3)$$

This equation can be rearranged to solve for N , the number of bound analyte molecules. The limit of detection (LOD) in terms of N , can then be determined by replacing $\Delta\lambda_{\max}$ in the equation with the minimum $\Delta\lambda_{\max}$ detectable by the optical detection system. The minimum $\Delta\lambda_{\max}$ detected depends on the optical system itself, data analysis method, and even the nanostructure since the amount of signal and peak width of $\lambda_{\max}$ depends on the nanostructure geometry.¹⁵⁴ As the signal, or scattering cross section, increases and the peak width decreases, the ability to detect $\Delta\lambda_{\max}$ improves, leading to a lower LOD.

One problem with the equations described above is that it does not take into account the non-homogeneous distribution of the electric field enhancement across a particular nanostructure. It is well known that sharp edges and corners of nanostructures

exhibit much larger electric field enhancement compared to smooth, or gradually curving regions.⁵⁸ For example, the electric field enhancement is large at the ends of nanorods, vertices of triangular nanoplates, points of branched structures, or within coupled nanoparticle pairs.^{58, 156} These regions are considered to be “hot spots” that are highly sensitive to molecules binding in the vicinity much in the same way that these “hot spots” are responsible for surface enhanced Raman scattering (SERS) of Raman active molecules in their vicinity. In terms of the equations above, the non-homogenous distribution of the regions of electric field enhancement leads to a much smaller effective V_s than would normally be calculated experimentally. V_s has been calculated previously by adsorbing polyelectrolyte layers onto the surface of Au nanorods and other nanostructures and the $\Delta\lambda_{\text{max}}$ monitored as a function of thickness.¹⁰² V_s is then defined as the volume completely surrounding the nanostructure out to a distance where the signal is 5% of the maximum.¹⁵⁴ This assumes that the polyelectrolyte molecules are all contributing to the signal equally, when in fact many of them adsorb to insensitive regions of the nanostructure and do not contribute appreciably to the signal. This leads to an overestimate of the V_s . If there is no control over the binding location of the analyte, though, the above analysis agrees well with the experimental results since analyte molecules have equal probability of binding outside or within “hot spots”. The overall average then matches theory with good agreement.

The goal of our work was to selectively bind analyte molecules to the “hot spots” of metal nanostructures in order to better understand the effect of binding location on the LSPR response and ultimately improve the limit of detection (LOD) for biosensing applications. As mentioned above, controlled binding to the sensitive regions of the

metal nanostructure effectively reduces V_s . The $\Delta\lambda_{\max}$ (or signal) will accordingly increase in equation 3. Even though the binding location is predicted to be an important factor in LSPR sensing, there are few experimental examples of controlled binding of analyte to “hot spots” or characterization of the analyte binding location on a nanostructure during LSPR sensing. While researchers have studied LSPR sensing with nanoparticles of various sizes and shapes, even down to the single particle level, there is still a lack of knowledge about how the LSPR response depends on the number of biomolecules binding and the location of the binding site on various size/shape metal nanostructures which are critical issues for optimizing the sensitivity of these types of biosensors. We have demonstrated experimentally that the edges of the nanostructures are very sensitive to protein binding by correlating the optical properties with Atomic Force Microscopy (AFM) images for on spherical Au nanoparticles and on the purified nanoplates for the first time as described in chapters III and V. We showed that edge-functionalized pure Au nanoplates showed pronounced optical responses to analyte binding which is discussed in chapter IV. After demonstrating that the edges are sensitive to protein binding, we optimized the sensing distance by attaching the proteins close to the nanoparticle surface by changing the linker which is described in chapter V. Chapter VI of the dissertation shows a detailed LSPR responses to specific vs nonspecific IgG on edge attached anti-IgG. In Chapter VII we extended the LSPR sensing strategies of proteins through selective edge functionalization to Ag metal and showed bigger optical responses to global changes than the corresponding Au nanoplates.

CHAPTER II

EXPERIMENTAL

2.1. Substrates

2.1.1 Silicon. Silicon substrates (Si/SiO_x) were purchased from Silicon Quest International. Si/SiO_x was used to grow the metal nanostructures directly on the surface for analysis by Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM).

2.1.2 Glass. Glass substrates were purchased from Corning. Glass slides were used to grow the metal nanostructures directly on the surface for analysis of their optical properties by UV-vis spectroscopy. These were used for all Localized Surface Plasmon Resonance (LSPR) studies. Glass slides were also cut and cleaned using the same procedure as silicon.

2.2. Synthesis of citrate capped Au nanoparticle “seeds”. An aqueous solution of Au nanoparticle “seeds” was prepared by adding 0.5 mL of 0.01 M sodium citrate trisodium salt and 0.05 mL of 0.01 M HAuCl₄ to 19 mL of water while stirring and then adding 0.6 mL of ice cold 0.1 M sodium borohydride while stirring. The seed solution was allowed to stir for at least 2 h prior to use. The resulting Au nanoparticle seeds are about 3-5 nm in size. These were later used for the seed-mediated growth of Au nanoplates. The seed solution is good to use after 2 hrs of its preparation and bad after 5 hrs.

2.3. Preparation of Growth Solution. We used two different growth solutions for metal nanostructure synthesis, which was prepared fresh each time. The growth solution 1 (GS 1) consists of 9 mL of 0.1 M cetyltrimethylammonium bromide (CTAB), 450 μ L of 0.01 M HAuCl₄, and 50 μ L of 0.01 M ascorbic acid. The growth solution 2 (GS2) consists of 9 mL of 0.016 M CTAB, 450 μ L of 0.01 M HAuCl₄, and 150 μ L of 0.2 M ascorbic acid. The source of the CTAB was Aldrich (95%) in both growth solutions, which was critical for synthesizing samples with a large population of nanoplates.⁵¹

2.4. Procedure for Cleaning Glass and Silicon Slides. Glass and silicon slides were first cut and cleaned in piranha solution (1:3 H₂O₂:H₂SO₄) for 10-15 min. **(Caution: this solution, piranha, is a strong oxidizing agent that reacts violently with organics.)** The slides were then rinsed with water and dried under a stream of N₂ before further use.

2.5. Functionalization of Glass and Silicon. The glass and silicon substrates were functionalized with mercaptopropyltrimethoxy silane (MPTMS) by heating them just below boiling in a solution containing 10 mL of 2-propanol, 100 μ L of MPTMS, and a few drops of water for about 30 min, then rinsed with 2-propanol and dried under N₂. This results in the silane groups attaching covalently to the glass and silicon surface and thiol groups terminating the surface for attachment of Au nanoparticle seeds later.

2.6. Synthesis of Au Nanostructures Directly on Surfaces. Au nanostructures were synthesized directly on glass microscope slides and Si/SiO_x surfaces by a seed-mediated growth procedure.⁴⁷ Figure 2.1 shows a scheme of the general procedure using the

procedures described in 2.1 to 2.5. The mercaptopropyltrimethoxy silane (MPTMS) functionalized silicon and glass slides were placed in an aqueous solution of 3-5 nm diameter citrate-stabilized Au nanoparticles (“seeds”) for 15 minutes, which leads to their attachment to the thiol functionality of MPTMS through a strong Au-thiolate interaction. After rinsing with water, the substrates containing immobilized Au seed nanoparticles were then placed in a freshly prepared solution known as growth solutions as described previously (GS1 and GS2). In both cases, this leads to the preferential reduction of Au from solution onto the surface-attached Au nanoparticle “seeds” *via* ascorbic acid, leading to the growth of the 3-5 nm diameter Au nanoparticles into larger Au nanostructures directly on the surface. The samples were rinsed with water and dried under nitrogen before further use.

2.7. Purification of Au nanoplates. The Au nanoplates synthesized by seed-mediated growth are ~ 23% and ~ 44% pure using GS1 and GS2, respectively. This requires further purification by tape or sonication. Both methods below lead to ~ 90% Au nanoplates on the surface.

2.7.1. Purification by tape. We used Scotch brand magic adhesive tape to preferentially remove the spherical nanoparticles from the glass or Si/SiO_x surface. The tape was placed on the substrate, pressed gently with one finger and then slowly peeled back at an approximately 90° angle. In the case of glass, the procedure was performed on both sides.

Figure 2.1. Scheme showing the general procedure for the synthesis of gold nanostructures directly on a surface. A) side view and B) top view.

2.7.2. Purification by sonication. We used a Branson ultrasonic cleaner with a 935 W input and 250 W output puissance HF to remove the spherical nanoparticles from substrates containing Au nanostructures. The substrate was placed in a glass vial containing 10 mL of nanopure water and then placed in the ultrasonicator for 2-5 minutes. The substrate was removed from the vial, washed thoroughly with nanopure water, and dried under nitrogen.

2.8. Synthesis of Ag-coated Au nanoplates (Au/Ag). Au nanoplates were synthesized first using the procedure described in 2.6 and then purified by ultrasonication of the sample. The resulting Au nanoplate sample was placed in an Ag growth solution containing 10 mL of 0.08 M CTAB in pH 10.6 phosphate buffer, 250 μ L of 0.01 M AgNO₃ and 500 μ L of 0.1 M ascorbic acid for 30 minutes.¹⁵⁷ The samples were then washed with nanopure water and dried under nitrogen.

2.9. Preparation of Anti-Human IgG and Human IgG. The stock solutions of anti-IgG and IgG were diluted in an aqueous solution of phosphate buffered saline, (PBS) whose pH is 7.4. The phosphate buffered saline (PBS) was purchased from Sigma-Aldrich and was dissolved in 1000 mL of nanopure water and used to prepare diluted solutions of anti-IgG and IgG. The resulting diluted solutions were stored in the refrigerator until further use.

2.10. Functionalizing the Au Nanostructures and Au films with Anti-IgG. Anti-IgG was immobilized on the Au nanostructures and Au films by two different linking

methods. The first method, termed EDC coupling, involved immobilization onto the Au nanoplates by placing the sample in a 1 mM ethanol solution of mercapto-carboxylic acid, mercaptoundecanoic acid (MUA) or mercaptopropionic acid (MPA) for 12-15 h, rinsing thoroughly with ethanol, drying under N₂, and then placing the sample in an aqueous solution of 2 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 5 mM N-hydroxy succinimide (NHS) for 1 h. After rinsing with water and drying under N₂, we placed the sample in an aqueous 0.029 to 0.29 µg/mL human anti-IgG solution for 12-15 h, rinsed with pH 7.4 phosphate buffered saline and water, and dried under N₂. Figure 2.2 shows a scheme for immobilizing anti-human IgG on the surface of Au nanostructures for IgG sensing.

The second method, termed DSNB coupling was performed by exposing the Au to a solution of 5, 5'-Dithiobis (succinimidyl-2-nitrobenzoate) (DSNB) as shown in Figure 2.3. Antibody immobilization occurs by placing the slides in antibody solution for 12-15 h, washing with pH 7.4 phosphate buffered saline to remove physisorbed antibody, washing with water and drying under nitrogen. The slides were then placed in an antigen solution specific for the antibody for 12-15 hrs.

2.11. IgG Sensing. Human IgG sensing was performed by placing the sample of human anti-IgG functionalized Au nanoplates and films in a pH 7.4 phosphate buffered aqueous saline solution containing human IgG overnight (~24 h) in the refrigerator before rinsing with pH 7.4 phosphate buffered saline and water and drying under N₂. Figure 2.2 shows

Figure 2.2. Procedure for covalently attaching anti-IgG to Au nanostructures functionalized with a pure monolayer of mercaptoundecanoic acid (MUA) for sensing IgG. We used three different variations of this procedure - Pure MUA, 10 % MUA and place-exchanged MUA which is discussed in more detail in chapter III.

Figure 2.3. Coupling strategy for antibody immobilization using DSNB as a linker.

a general scheme for sensing IgG (antigen) after immobilizing anti-human IgG (antibody) onto Au nanostructures.

2.12. Determining Conditions for Anti-IgG and IgG Sensing. Anti-IgG immobilization on Au nanoplates was confirmed by Atomic Force Microscopy (AFM) images as shown in Figure 2.4 at various concentrations of 2.9 $\mu\text{g/mL}$ (Frame A), 0.29 $\mu\text{g/mL}$ (Frame B), 0.029 $\mu\text{g/mL}$ (Frame C), 0.0029 $\mu\text{g/mL}$ (Frame G) and 0.00029 $\mu\text{g/mL}$ (Frame H) and the corresponding zoom-in images. We confirmed antibody/antigen immobilization by 1) imaging nanoplates with AFM as described above and 2) monitoring the surface FTIR spectra of Au films as described next. Although we did not use DSNB coupling for all experiments in chapters III-VII it was used as a proof of concept that antibody was successfully immobilized onto the Au surface through covalent linkage. We observed some nonspecific adsorption by observing the optical properties on Au nanoplate samples at concentrations of 2.9 $\mu\text{g/mL}$ anti-IgG and higher. FTIR data also confirmed this since we saw strong absorption bands around 1600-1700 cm^{-1} and 1500-1600 cm^{-1} corresponding to the amide I and amide II bonds of antiIgG on samples with no linker and control experiments using butanethiol as a linker instead of carboxylic acid thiols as shown in Figure 2.5. This lead us to use 0.29 and 0.029 $\mu\text{g/mL}$ anti-IgG for all experiments in chapters III-VII, where we noticed no change in optical responses in control experiments with mercaptoethanol (ME) treated Au nanoplate samples.

Figure 2.4. AFM images of Au nanoplates functionalized using the procedure in Figure 2.2 with anti-IgG concentrations of A) 2.9 $\mu\text{g/mL}$ B) 0.29 $\mu\text{g/mL}$ and C) 0.029 $\mu\text{g/mL}$. (D), (E), and (F) are the corresponding expanded images.

Figure 2.4 continued. AFM images of Au nanoplates functionalized using the procedure in Figure 2.2 with anti-IgG concentrations of G) 0.0029 $\mu\text{g/mL}$ H) 0.00029 $\mu\text{g/mL}$. (I), (J) are the corresponding expanded images.

Figure 2.5. FTIR spectra showing response for control experiments using butanethiol (BTH) and no linker before immobilizing anti-IgG.

2.13. Instrumentation

2.13.1. UV-vis spectroscopy. UV-vis spectra were obtained with a Varian CARY 50 BIO UV-vis spectrophotometer. UV-VIS spectroscopy was used to measure the optical properties of the nanostructures (Au nanoplates) on the surface of the glass. This provided information about the composition and shape of the nanostructures and optical changes during antibody/antigen attachment. We reduced the noise in all of the extinction spectra by using the smooth operation in the Varian software with a filter size of 101. Figure 2.6.A shows a typical UV-vis spectra of the as synthesized Au nanostructures using growth solution 1.

2.13.2. Atomic Force Microscopy.

Atomic force microscopy (AFM) images were obtained with a Veeco Digital Instruments Nanoscope IIIa Multimode scanning probe microscope (Santa Barbara, CA) using a Si tip in tapping mode. AFM provides the topography of the sample giving information about the size, shape and coverage of the immobilized nanostructures. Figure 2.6.B shows an AFM image of the synthesized Au nanostructures on the surface of silicon. AFM was also used to image antibody anti-IgG on the surface as shown in Figure 2.5 and also provides information about nanoplate height in order to calculate the aspect ratio, which was not possible by SEM.

2.13.3. Scanning Electron Microscopy.

Scanning electron microscopy (SEM) images were obtained with a FEI-NOVA-600 NANO SEM. SEM provides information about the size, shape and coverage of the Au

Figure 2.6. A) UV-vis spectra of the nanostructures synthesized on glass by GS1 as-prepared. B) AFM and C) SEM images of the nanostructures on a silicon surface.

nanoplates synthesized directly on surfaces. Figure 2.6.C shows an SEM image of the synthesized Au nanostructures on a silicon surface. SEM provides a clear image, which is useful for width and shape measurements of nanostructures.

2.13.4. Fourier Transform Infrared Spectroscopy.

We used a Digilab-Win IR to record the surface reflectance infrared spectra of antibody and antigen attached to Au films. Figure 2.7 shows a cartoon of the antibody attachment on an Au film using mercaptocarboxylic acid as a linker. FTIR initially confirmed that our antibody/antigen coupling strategy was successful. We characterized Au films by surface FTIR before and after antibody and antigen attachment. Figure 2.8.A shows the FTIR spectra of anti-IgG immobilized by EDC/NHS coupling on an Au film functionalized with mercaptopropionic acid (MPA) and Figure 2.9. A shows the spectra of anti-IgG immobilization using DSNB as a linker. The spectrum after antiIgG shows strong absorption bands around $1600\text{--}1700\text{ cm}^{-1}$ and $1500\text{--}1600\text{ cm}^{-1}$ corresponding to the amide I and amide II bonds of antiIgG. Antigen specific for antiIgG is detected by an increase in absorption at around $1500\text{--}1700\text{ cm}^{-1}$, which corresponds to the additional amide bonds of the IgG. Figure 2.8 B and 2.9. B shows the FTIR data for specific and nonspecific antigen. The amide I and amide II bands at $1600\text{--}1700\text{ cm}^{-1}$ and $1500\text{--}1600\text{ cm}^{-1}$ increased for the specific antigen IgG while they remained the same for nonspecific antigen. FTIR data served as a proof of concept that antibody can be immobilized on the surface of Au through covalent linkage using the two methods as described above. It also showed specific interaction between anti-IgG and the corresponding antigen IgG. The FTIR on Au films helped us choose the concentrations and conditions for functionalizing

Au nanoplates with antibody/antigen. We eventually chose terminated COOH thiols and EDC coupling and anti-IgG concentration of 0.29 $\mu\text{g/ml}$ or less to avoid non-specific adsorption.

Figure 2.7. Cartoon showing antibody immobilization on the surface of an Au film for FTIR characterization.

Figure 2.8. A) FTIR spectra of antibody antiIgG immobilized on an Au film by EDC coupling to mercaptopropionic acid and detection of IgG antigen. B) FTIR spectra of an anti-IgG functionalized Au film exposed to catalase (non-specific) and IgG (specific).

Figure 2.9. A) FTIR spectra of antiIgG immobilized on an Au film functionalized with DSNB and detection of IgG antigen. B) FTIR spectra of anti-IgG functionalized Au film after exposure to non-specific catalase.

CHAPTER III

SELECTIVE ATTACHMENT OF ANTIBODIES TO THE EDGE SITES OF GOLD NANOSTRUCTURES FOR ENHANCED LOCALIZED SURFACE PLASMON RESONANCE BIOSENSING

Here we demonstrate control over the location of anti-IgG proteins bound to Au nanoplates formed on glass and silicon samples. Preferential attachment to edge and vertex sites occurs by performing a thiol place-exchange reaction between mercaptoethanol (ME) attached to the Au nanostructures and mercaptoundecanoic acid (MUA) in solution, which localizes the carboxylic acid groups of MUA on the edge sites for subsequent amide linkage of anti-IgG. In contrast, anti-IgG attaches randomly onto the terrace regions of Au nanostructures functionalized directly in pure MUA or 1:10 MUA:ME solutions. Importantly, Au nanostructures with anti-IgG selectively bound to the edge sites exhibit significantly larger changes in the absorbance and wavelength of maximum absorbance (λ_{max}) of their localized surface plasmon resonance (LSPR) response upon binding compared to those with anti-IgG randomly attached to terrace regions. This leads to at least 500 times more sensitive detection of IgG down to 0.1 ng/mL.

3.1. INTRODUCTION

Localized surface plasmon resonance (LSPR) is the result of collective oscillations of conduction band electrons for noble metals like Au, Ag.⁶⁷⁻⁷¹ The wavelength of maximum absorption depends on size, shape, composition, environment surrounding the metal nanoparticle.⁶⁷⁻⁷¹ LSPR can be used for sensing by monitoring the changes in the LSPR band when analyte bounds to the receptor attached to the nanoparticle surface. The sensor responds either by a red shift in the LSPR band to longer wavelengths or increase in intensity of the LSPR band of the metal nanoparticles upon binding of analyte. LSPR based sensing is low cost, simple and label-free method. Van Duyne and co-workers predicted that the edges of nanostructures are very sensitive to local changes while terrace sites are less sensitive.¹⁰⁷ While the existence of sharp edges are often used to explain different LSPR sensitivities for different-shaped nanoparticles, there have been no reports demonstrating controlled binding of analyte to the edge regions of metal nanostructures or providing detailed characterization of the binding location. This chapter describes selective attachment of antibodies to the edges of Au nanostructures demonstrating enhanced LSPR response versus terrace attached antibodies.

3.2. EXPERIMENTAL

Details on the Synthesis of Au Nanostructures

Au nanostructures were synthesized directly on glass and silicon surfaces by a seed-mediated growth procedure described first by Murphy and co-workers for the solution-phase synthesis of Au nanorods¹⁵⁸ and later by Taub *et al.*,¹⁵⁹ our group,¹⁶⁰⁻¹⁶⁴ and others¹⁶⁵ for the synthesis of Au nanorods and other structures directly on surfaces. Glass and silicon slides were first cut and cleaned in piranha solution (1:3 H₂O₂:H₂SO₄) for 10-15 min. After rinsing with water and drying under a stream of N₂, the substrates were functionalized with mercaptopropyltrimethoxy silane (MPTMS) by heating them just below boiling in a solution containing 10 mL of 2-propanol, 100 μ L of MPTMS, and a few drops of water for about 30 min. After rinsing with 2-propanol and drying under N₂, The MPTMS functionalized silicon and glass slides were placed in an aqueous solution of 3-5 nm diameter citrate-stabilized Au nanoparticles (“seeds”)¹²¹ for 15 minutes, which leads to their attachment to the thiol functionality of MPTMS through a strong Au-thiolate interaction. After rinsing with water, the substrates containing immobilized Au seed nanoparticles were then placed in a freshly prepared solution containing 9 mL of 0.1 M cetyltrimethylammonium bromide (CTAB), 450 μ L of 0.01 M HAuCl₄, and 50 μ L of 0.01 M ascorbic acid for 1 h. This leads to the preferential reduction of Au from solution onto the surface-attached Au nanoparticle “seeds” via ascorbic acid, leading to the growth of the 3-5 nm diameter Au nanoparticles into larger Au nanostructures directly on the surface.^{163, 164} The samples were rinsed with water and dried under nitrogen before further use. We previously reported on the use of this method to grow 10-15% Au nanorods directly on the surface.¹⁶²⁻¹⁶⁴ Recent reports show that the growth of nanorods

depends on the source of the CTAB.¹⁶⁶ In this case, we used CTAB from Aldrich Chemical (98%), which led to a large percentage of Au nanoplates on the surface as opposed to nanorods for reasons that are not understood. For this research, we desired a large number of Au nanoplates in order to monitor the coverage and location of the anti-IgG bound to the nanostructures as discussed later. Figure 3.1 shows an AFM image of a typical silicon sample, which consisted of 67% spherical particles, 27% triangular nanoplates, and 6% circular nanoplates. The nanoplates were in the range of 100 – 200 nm on a side.

Schemes Showing the Procedure for Functionalizing the Au Nanostructures with Anti-IgG

We covalently attached anti-IgG to the Au nanostructures by three methods. In the first method, termed “pure MUA”, we placed the sample in a 1 mM ethanol solution of mercaptoundecanoic acid (MUA) for 12-15 h, rinsed thoroughly with ethanol, dried under N₂, and then placed the sample in an aqueous solution of 2 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 5 mM N-hydroxy succinimide (NHS) for 1 h. After rinsing with water and drying under N₂, we placed the sample in an aqueous 0.029 or 0.29 µg/mL human anti-IgG solution for 12-15 h, rinsed with phosphate buffered saline and water, and dried under N₂. The second procedure, termed “10% MUA”, involved the same procedure, except that we placed the sample into an ethanol solution containing a 1:10 mole ratio (total of 1 mM) of MUA and mercaptoethanol (ME) prior to anti-IgG coupling. We term this 10% MUA due to the solution composition, but the monolayer composition is likely different. In the third procedure, termed “place-

Figure 3.1. An atomic force microscopy (AFM) image of (A) Au nanoplates and other nanostructures grown on a silicon surface and (B) expanded image of an Au nanoplate.

exchange”, we placed the sample in a 1 mM ethanol solution of ME overnight and then exchanged the ME monolayer with MUA by placing the sample into a 5 mM ethanol solution of MUA for 4 h. Finally, we attached anti-IgG via EDC and NHS coupling. We hypothesized that the place-exchange strategy would lead to selective edge functionalization based on results by Murray and co-workers, who speculated that thiol place-exchange reactions on Au nanoparticles occur preferentially at vertex and edge sites due to less steric hindrance. If true, the MUA molecules should exchange at edge and vertex sites on the Au nanoplates and nanoparticles, which would subsequently lead to the attachment of anti-IgG to these sites.

Figure 3.2 shows the first two methods (“pure MUA” and “10% MUA”) used to covalently attach anti-IgG to the surfaces of the Au nanostructures. These two strategies lead to attachment of anti-IgG onto the terraces of Au nanoplates (and presumably Au nanoparticles) through an amide linkage. Figure 3.3 shows our strategy for edge functionalization of Au nanoplates (and presumably Au nanoparticles) with anti-IgG by “place-exchange”.

Figure 3.2. Schematic procedure for covalently attaching anti-IgG to Au nanostructures functionalized with A) a pure monolayer of mercaptoundecanoic acid (MUA) – “pure MUA” and B) a mixed monolayer of MUA and mercaptoethanol (1:10 ratio) – “10% MUA”.

Figure 3.3. Schematic procedure for selective edge attachment of anti-IgG to Au nanoplates – “place-exchange”.

3.3. RESULTS AND DISCUSSION

AFM Images of Anti-IgG on Au Nanoplates

Figure 3.4 shows AFM images of Au nanoplates functionalized by the three strategies at the two different concentrations of 0.29 $\mu\text{g/mL}$ and 0.029 $\mu\text{g/mL}$ anti-IgG. We focused on Au nanoplates because they are atomically smooth and allow easy visualization of anti-IgG on the surface. In contrast, we could not identify anti-IgG on the highly curved spherical nanoparticles by AFM. We assume that the coverage and location of anti-IgG on the Au nanoplates reflects that on the spherical nanoparticles. We assign the bright spots on the nanoplates in Figure 3.4 to the attached anti-IgG. For the pure MUA strategy (Figure 3.4A and 3.4B), the coverage decreased as the concentration of the anti-IgG decreased. This also occurred for the 10% MUA strategy (Figure 3.4C and 3.4D), but the coverage was lower at both concentrations compared to pure MUA due to a fewer number of binding sites on the surface. At 0.029 $\mu\text{g/mL}$, the 10% MUA surface does not show any bound anti-IgG. With both strategies, the anti-IgG bound randomly to the flat terrace sites of the nanoplates. In contrast, for the place-exchange sample (Figure 3.4E and 3.4F), the anti-IgG attached preferentially at the edges, and even the vertex in Figure 3.4F, of the Au nanoplates. The coverage did not correlate well with anti-IgG concentration when comparing 0.29 and 0.029 $\mu\text{g/mL}$, suggesting that it was limited by the amount of MUA exchanged on the edge. While only two images are shown in Figure 3.4, edge localization occurred for 19 out of 22 nanoplates imaged at the two concentrations (See Figure 3.5 and 3.6 for AFM images of other nanoplates). As

Figure 3.4. AFM images of Au nanoplates functionalized with anti-IgG using the (A, B) pure MUA, (C, D) 10% MUA, and (E-H) place-exchange strategies and anti-IgG concentrations of (A, C, E, G, H) 0.29 $\mu\text{g/mL}$ and (B, D, F) 0.029 $\mu\text{g/mL}$. Place-exchange occurred with (E, F) 5 mM MUA and (G, H) 6 mM MUA.

further evidence that selective edge functionalization occurred, increasing the MUA concentration to 6 mM during the place-exchange reaction led to more anti-IgG on the edge sites as shown in Figure 3.4G and 3.4H (See Figure 3.7 also). We measured the root-mean-square (RMS) roughness of the Au nanoplates as a function of anti-IgG concentration (Figure 3.8) to quantify the surface coverage of anti-IgG for the three strategies. The pure MUA strategy had the largest anti-IgG coverage since it had the most MUA binding sites on terraces.

Figure 3.5 shows 11 AFM images of Au nanoplates with anti-IgG attached by the place-exchange method using a 0.029 $\mu\text{g/mL}$ concentration. The anti-IgG clearly attached to edge sites in 9 of the nanoplates. Figure 3.6 shows another 11 AFM images of Au nanoplates with anti-IgG attached by the place-exchange method using a 0.29 $\mu\text{g/mL}$ concentration. The anti-IgG attached to edge sites in 10 out of these 11 nanoplates. Only 3 nanoplates out of the 22 plates imaged did not show edge functionalization; there was no visible anti-IgG on the surface. We believe this serves as proof that preferential edge attachment occurred by our method. Figure 3.7 shows 5 images where we attached anti-IgG at a concentration of 0.29 $\mu\text{g/mL}$ after place-exchange with 6 mM MUA instead of 5 mM. In 4 out of the 5 images, the edge attachment of anti-IgG is enhanced versus terrace sites. This further supports that selective edge attachment occurs.

Figure 3.5. AFM images of anti-IgG bound to Au nanoplates using the place-exchange method with an anti-IgG concentration of $0.029 \mu\text{g/mL}$ and 5 mM MUA concentration during exchange. Nine out of the 11 nanoplates clearly had anti-IgG attached to edge sites.

Figure 3.6. AFM images of anti-IgG bound to Au nanoplates using the place-exchange method with an anti-IgG concentration of 0.29 $\mu\text{g/mL}$ and 5 mM MUA concentration during exchange. Ten out of 11 nanoplates show edge attachment of anti-IgG.

Figure 3.7. AFM images of anti-IgG bound to Au nanoplates using the place-exchange method with an anti-IgG concentration of $0.29 \mu\text{g/mL}$ and 6 mM MUA concentration during exchange. While not all of the anti-IgG are on edge sites, most are and the number of anti-IgG bound to edges increased due to the increasing MUA concentration during exchange.

Root-Mean-Square (RMS) Roughness Measurements to Quantify Anti-IgG Coverage

Figure 3.8. RMS roughness measurements as a function of anti-IgG concentration for the different strategies as shown. The roughness ranges from about 0.07 nm for no anti-IgG adsorption to about 0.75 nm for a full coverage of anti-IgG. The larger the RMS value the larger the anti-IgG coverage.

It was very difficult to count individual anti-IgG on the Au nanoplates to determine a coverage in terms of the number of anti-IgG per nanoplate or number of anti-IgG per unit area. Instead, we measured the RMS roughness of the middle region of the Au nanoplates, excluding the edges, to indirectly quantify the coverage. As the coverage of anti-IgG on the Au nanoplates increases, the RMS roughness increases, giving an indirect measurement of coverage. Figure 3.8, Frame A, shows the RMS roughness measurement of Au nanoplates functionalized by the pure MUA method as a function of anti-IgG concentration from 0 $\mu\text{g/mL}$ up to 2.9 $\mu\text{g/mL}$. The 0 $\mu\text{g/mL}$ represents the average roughness of bare Au nanoplates (RMS ~ 0.07 nm) and 2.9 $\mu\text{g/mL}$ represents an approximate full coverage of anti-IgG (RMS ~ 0.75 nm) on the surface based on AFM images (not shown). This shows that the coverage of the Au nanoplates can be controlled by the anti-IgG concentration. We found that there was also some non-specific adsorption at 2.9 $\mu\text{g/mL}$ for the Au nanoplates. For this reason, we did not include AFM and spectroscopy data for the different strategies at this concentration. Figure 3.8, Frame B, shows the RMS roughness of Au nanoplates functionalized by the various strategies as a function of the two anti-IgG concentrations. Pure ME is a control sample, where anti-IgG can only adsorb by non-specific adsorption since no covalent attachment is possible. At 0.029 $\mu\text{g/mL}$, the pure ME, 10% MUA, and place-exchange samples all show RMS roughness values similar to bare Au nanoplates. This is consistent with the AFM images of the 10% MUA, which showed no anti-IgG attachment, and the fact that there is no non-specific adsorption on ME-coated surfaces. The place-exchange has a similar RMS roughness as bare Au because it is measured in the middle of the Au nanoplate, where there is no anti-IgG since it is located preferentially on the edges. Only pure MUA has a

significant roughness at 0.029 $\mu\text{g/mL}$ because there is a significant amount of anti-IgG attached to the terraces at this concentration. At 0.29 $\mu\text{g/mL}$, both pure MUA and 10% MUA have significant RMS roughness values since they both contain anti-IgG on the terrace regions. The place-exchange surface again shows the same roughness of bare Au because the anti-IgG is located on the edges. The pure ME Au nanoplates have the same roughness as bare Au because there is very little or no non-specific adsorption of anti-IgG on the surface at this concentration.

Visible Spectra Before and After Anti-IgG Binding

We used a Varian Cary 50 Bio UV-vis spectrophotometer to monitor the absorbance in air (in transmission mode) of the Au nanostructures grown on glass substrates before and after attachment of anti-IgG by the three different strategies. Figure 3.9 shows the visible spectra of samples of Au nanostructures functionalized by the pure MUA (3.9A and 3.9B), 10% MUA (3.9C and 3.9D), and place-exchange (3.9E and 3.9F) methods before and after attachment of anti-IgG from 0.29 and 0.029 $\mu\text{g/mL}$ concentrations (See Figures 3.10-3.18 for all samples). Each sample displayed a major LSPR band between 530 and 550 nm attributed to the Au nanostructures (mainly spherical nanoparticles). We normalized each pair of spectra to the absorbance at the wavelength of maximum absorbance (λ_{max}) before anti-IgG attachment, which made the absorbance at λ_{max} equal to 1.0 before anti-IgG attachment and allowed easy comparison of the relative absorbance increase after anti-IgG attachment for each strategy. Qualitatively, for the pure MUA and 10% MUA samples, there was no significant increase in the LSPR intensity or wavelength due to binding of anti-IgG from the 0.29 and 0.029 $\mu\text{g/mL}$ solutions; the

Figure 3.9. Visible spectra of Au nanostructures functionalized with A-B) pure MUA, C-D) 10% MUA, and E-F) place-exchange strategy before and after attachment of anti-IgG from concentrations of A, C, E) 0.29 $\mu\text{g/mL}$ and B, D, F) 0.029 $\mu\text{g/mL}$. Inset shows zoom-in of peaks.

absorbance actually decreased in 2 out of the 4 cases. In contrast, there was a significant increase in the intensity and λ_{max} upon binding of anti-IgG from both the 0.29 and 0.029 $\mu\text{g/mL}$ solutions for the place-exchange samples. Based on the AFM images, we believe this is due to anti-IgG being located at edge or vertex sites for the place-exchange samples. Even though the anti-IgG coverage is comparably larger for the pure MUA at both concentrations and the 10% MUA at 0.29 $\mu\text{g/mL}$, the optical response is insignificant because the anti-IgG is located on the less sensitive terraces. This qualitatively confirms that the LSPR response of Au nanostructures is more sensitive to dielectric changes (analyte binding) on edge sites as compared to terrace sites. The Van Duyne group previously calculated that a Ag nanotriangle could be less than 10% covered, but still exhibit almost its maximum shift if the adsorbate is confined to the edges.

Figure 3.10 shows a quantitative statistical analysis of the change in λ_{max} ($\Delta\lambda_{\text{max}}$) and change in normalized absorbance ($\Delta A/A_{\text{initial}}$) as a function of the two anti-IgG concentrations for the three different functionalization strategies and for Au functionalized with ME only as a control (See Table 3.1 and 3.2 for all data). ΔA is equal to $A_{\text{final}} - A_{\text{initial}}$, where A_{initial} and A_{final} are the absorbances at λ_{max} before and after anti-IgG binding, respectively. The bar graphs show the average and standard deviation measured from 3 samples of each strategy (2 for pure ME). We performed the measurements by marking the samples and monitoring the same area of the glass slide before and after anti-IgG binding. The deviation in $\Delta\lambda_{\text{max}}$ and normalized $\Delta A/A_{\text{initial}}$ on an untreated sample over a one-week period was at most 2 nm and 0.02 (2%), respectively. Signals equal to or below these values in Figure 3.10 are therefore

Figure 3.10. Bar graphs of A) $\Delta\lambda_{\max}$ and B) $\Delta A/A_{\text{initial}}$ for both anti-IgG concentrations using the three different strategies and ME only.

Spectroscopy Data Tables for the Different Strategies and Anti-IgG Concentration

Table 3.1. Data table showing the absorbance for all samples before (A_{initial}) and after (A_{final}) attachment of anti-IgG as a function of attachment strategy and anti-IgG concentration. Table also shows the absolute (ΔA) and normalized absorbance change ($\Delta A/A_{\text{initial}}$).

6 mM MUA place-exchange					
Concentration	Sample	A_{initial}	A_{final}	ΔA	$\Delta A/A_{\text{initial}}$
0.29 $\mu\text{g/mL}$	1	0.072	0.077	0.005	0.069
	2	0.066	0.073	0.007	0.106
	3	0.071	0.081	0.01	0.141
Average					0.105
Std. Dev.					0.036
5 mM MUA place-exchange					
Concentration	Sample	A_{initial}	A_{final}	ΔA	$\Delta A/A_{\text{initial}}$
0.29 $\mu\text{g/mL}$	1	0.076	0.080	0.004	0.053
	2	0.053	0.067	0.014	0.264
	3	0.072	0.079	0.007	0.097
Average					0.138
Std. Dev.					0.112
0.029 $\mu\text{g/mL}$					
	1	0.087	0.092	0.005	0.057
	2	0.073	0.083	0.010	0.137
	3	0.084	0.096	0.012	0.143

Average					0.112
Std. Dev.					0.048
Pure MUA					
Concentration	Sample	A_{initial}	A_{final}	ΔA	ΔA/A_{initial}
0.29 μg/mL	1	0.056	0.055	-0.001	-0.018
	2	0.040	0.040	0.000	0.000
	3	0.105	0.104	-0.001	-0.010
Average					-0.009
Std. Dev.					0.009
0.029 μg/mL	1	0.140	0.135	-0.005	-0.036
	2	0.062	0.062	0.000	0.000
	3	0.047	0.048	0.001	0.021
Average					-0.005
Std. Dev.					0.029
1:10 MUA:ME					
Concentration	Sample	A_{initial}	A_{final}	ΔA	ΔA/A_{initial}
0.29 μg/mL	1	0.101	0.099	-0.002	-0.020
	2	0.085	0.084	-0.001	-0.012
	3	0.088	0.086	-0.002	-0.023
Average					-0.018
Std. Dev.					0.006

0.029 $\mu\text{g/mL}$	1	0.088	0.087	-0.001	-0.011
	2	0.070	0.065	-0.005	-0.071
	3	0.062	0.061	-0.001	-0.016
Average					-0.033
Std. Dev.					0.033

ME only					
Concentration	Sample	A_{initial}	A_{final}	ΔA	$\Delta A/A_{\text{initial}}$
0.29 $\mu\text{g/mL}$	1	0.051	0.047	-0.004	-0.078
	2	0.058	0.057	-0.001	-0.017
Average					-0.048
Std. Dev.					0.043
0.029 $\mu\text{g/mL}$	1	0.019	0.019	0	0.000
	2	0.018	0.018	0	0.000
Average					0.000
Std. Dev.					0.000

Table 3.2. Data table showing the wavelength of maximum absorbance for all samples before ($\lambda_{\max, \text{initial}}$) and after ($\lambda_{\max, \text{final}}$) attachment of anti-IgG as a function of attachment strategy and anti-IgG concentration. The change in $\lambda_{\max}$ is also shown ($\Delta\lambda_{\max}$)

6 mM MUA place-exchange				
Concentration	Sample	$\lambda_{\max, \text{initial}}$	$\lambda_{\max, \text{final}}$	$\Delta\lambda_{\max}$
0.29 $\mu\text{g/mL}$	1	548.0	558.0	10
	2	541.0	550.0	9
	3	547.0	556.0	9
				9.3
				0.6
5 mM MUA place-exchange				
Concentration	Sample	$\lambda_{\max, \text{initial}}$	$\lambda_{\max, \text{final}}$	$\Delta\lambda_{\max}$
0.29 $\mu\text{g/mL}$	1	536.0	544.0	8.0
	2	559.0	561.0	2.0
	3	545.0	552.0	7.0
	Average			5.7
	Std. Dev.			3.2
0.029 $\mu\text{g/mL}$	1	544.0	551.0	7.0
	2	544.0	548.0	4.0
	3	536.0	540.0	4.0
	Average			5.0

Std. Dev.				1.7
Pure MUA				
Concentration	Sample	$\lambda_{\max, \text{initial}}$	$\lambda_{\max, \text{final}}$	$\Delta\lambda_{\max}$
0.29 $\mu\text{g/mL}$	1	532.0	534.0	2.0
	2	556.0	556.0	0.0
	3	530.0	532.0	2.0
Average				1.3
Std. Dev.				1.2
0.029 $\mu\text{g/mL}$	1	546.9	547.9	1.0
	2	527.0	526.0	-1.0
	3	534.0	533.0	-1.0
Average				-0.3
Std. Dev.				1.2
1:10 MUA:ME				
Concentration	Sample	$\lambda_{\max, \text{initial}}$	$\lambda_{\max, \text{final}}$	$\Delta\lambda_{\max}$
0.29 $\mu\text{g/mL}$	1	523.0	523.0	0.0
	2	553.0	557.0	4.0
	3	542.0	541.0	-1.0
Average				1.0
Std. Dev.				2.6
0.029 $\mu\text{g/mL}$	1	552.0	551.0	-1.0

	2	544.1	542.1	-2.0
	3	541.1	537.1	-4.0
Average				-2.3
Std. Dev.				1.5
ME Only				
Concentration	Sample	$\lambda_{\max, \text{initial}}$	$\lambda_{\max, \text{final}}$	$\Delta\lambda_{\max}$
0.29 $\mu\text{g/mL}$	1	531.0	533.0	2.0
	2	530.0	527.0	-3.0
Average				-0.5
Std. Dev.				3.5
0.029 $\mu\text{g/mL}$	1	520.6	520.6	0
	2	520.7	519.3	-1.4
Average				-0.7
Std. Dev.				1.0

insignificant. Figure 3.10 shows that $\Delta\lambda_{\text{max}}$ and $\Delta A/A_{\text{initial}}$ were not significant or decreased for the pure MUA, 10% MUA, and ME samples at both concentrations. The decrease in $\Delta A/A_{\text{initial}}$ in some cases is due to loss of Au from the slide during soaking in anti-IgG. In contrast, the 5 mM place-exchange samples showed a significant positive $\Delta\lambda_{\text{max}}$ of 5-6 nm and a $\Delta A/A_{\text{initial}}$ greater than 0.10 (10%) at both concentrations. The 6 mM MUA exchange samples showed an even larger $\Delta\lambda_{\text{max}}$ of 9-10 nm for the 0.29 $\mu\text{g/mL}$ concentration (no data at 0.029 $\mu\text{g/mL}$), consistent with the AFM images showing more anti-IgG on the edges (Figure 3.4G and 3.4H). The $\Delta A/A_{\text{initial}}$ was not consistent with this at 6 mM likely because of the unpredictable amount of Au lost from the surface during soaking that competes with the absorbance increase due to anti-IgG binding. Regardless, these results clearly indicate that the place-exchange method leads to Au nanostructures significantly more sensitive to anti-IgG binding.

Since the Au nanoplates occupy only ~33% of the surface, the spherical nanoparticles dominate the visible spectrum, especially at 530-550 nm. We believe the AFM images of Au nanoplates can still explain the optical results, because they likely reflect the coverage and location of anti-IgG on the spherical particles as well, which are known to have well-defined geometries with edge and vertex sites. The edge sites are likely more sensitive for all nanoparticle shapes. We also note that the place-exchange samples often exhibited a positive baseline shift upon binding of anti-IgG for some of the samples (Figure 3.9F and Figure 3.16-3.18), showing that the absorbance increased in spectral regions outside the LSPR band. This baseline shift is a real effect of anti-IgG binding and not a baseline correction issue. All samples were referenced versus bare glass. In the 16 samples that were not sensitive to anti-IgG (pure MUA, 10% MUA, and ME), the

baseline did not increase significantly while 8 out of the 9 place-exchange samples did increase, showing that it is a real effect of anti-IgG binding for those samples.

Figures 3.11 to 3.18 show all of the visible spectra obtained in this study using the various anti-IgG attachment strategies and the control of pure ME. We focused on the spectral changes for the major LSPR response between 530 – 560 nm. We observed wavelength increases, absorbance increases, and sometimes positive baseline shifts for the place-exchange samples, which we believe is a real effect of anti-IgG binding to edge sites for those samples. We note that others often report a positive baseline shift upon analyte binding using LSPR.⁹⁵ In comparison, very little changes in wavelength or absorbance occurred for the other samples. If anything, the absorbance decreased slightly due to loss of some Au from the sample or the wavelength shifted negative for unknown reasons. There were sometimes small baseline, absorbance, or wavelength shifts at higher wavelengths, which are not clear at this time, but could be due to different shaped nanostructures on the surface. We focused on the major surface plasmon band at 530 – 560 nm, which mainly reflects changes occurring on spherical Au nanoparticles.

Pure MUA Samples

Figure 3.11. Visible spectra of three different samples of Au nanostructures functionalized with anti-IgG using the pure MUA method and 0.029 µg/mL anti-IgG.

Figure 3.12. Visible spectra of three different samples of Au nanostructures functionalized with anti-IgG using the pure MUA method and 0.29 µg/mL anti-IgG.

10% MUA Samples

Figure 3.13. Visible spectra of three different samples of Au nanostructures functionalized with anti-IgG using the 10% MUA method and 0.029 µg/mL anti-IgG.

Figure 3.14. Visible spectra of three different samples of Au nanostructures functionalized with anti-IgG using the 10% MUA method and 0.29 µg/mL anti-IgG.

Pure ME Samples

Figure 3.15. Visible spectra of two different samples of Au nanostructures functionalized with pure ME and exposed to 0.029 $\mu\text{g/mL}$ anti-IgG.

Figure 3.16. Visible spectra of two different samples of Au nanostructures functionalized with pure ME and exposed to 0.29 $\mu\text{g/mL}$ anti-IgG.

Place-exchange Samples (5 mM MUA)

Figure 3.17. Visible spectra of three different samples of Au nanostructures functionalized with anti-IgG using the place-exchange method (5 mM MUA) and 0.029 µg/mL anti-IgG.

Figure 3.18. Visible spectra of three different samples of Au nanostructures functionalized with anti-IgG using the place-exchange method (5 mM MUA) and 0.29 µg/mL anti-IgG.

Place-exchange Samples (6 mM MUA)

Figure 3.19. Visible spectra of three different samples of Au nanostructures functionalized with anti-IgG using the place-exchange method (6 mM MUA) and 0.29 $\mu\text{g/mL}$ anti-IgG.

Optical Detection of IgG

Figure 3.20 shows the visible spectrum of Au nanostructures grown on glass before and after human anti-IgG attachment using the place-exchange method with 5 mM MUA and 0.29 $\mu\text{g/mL}$ anti-IgG and also after exposure to 0.1 ng/mL human IgG solution for 24 h. The $\Delta\lambda_{\text{max}}$ and $\Delta A/A_{\text{initial}}$ are 8 nm and 11.0%, respectively, after anti-IgG attachment, consistent with other samples reported here. After human IgG exposure, $\Delta\lambda_{\text{max}}$ is negative, but the $\Delta A/A_{\text{initial}}$ changed by about 9.7%, which is significant. The change ranged from 6 – 10% for four samples. We do not fully understand the positive baseline shift, but note that it is a real effect of IgG binding based on the fact that no absorbance or baseline change occurred for human IgG exposure to samples functionalized with anti-IgG by the pure MUA and 10% MUA strategies or for the place-exchange samples exposed to non-specific goat IgG. Others also report positive baseline shifts in LSPR biosensing.⁹⁵ The 0.1 ng/mL concentration corresponds to ~ 7 pM, which is among the lowest detection limits reported for LSPR sensing. In comparison, samples functionalized with anti-IgG using the pure MUA and 10% MUA with a much higher coverage (2.9 $\mu\text{g/mL}$ anti-IgG) did not show a response to IgG until ~ 50 ng/mL, which is 500 times larger. Since the optical response is mainly from the spherical nanoparticles, we believe we can achieve much lower detection limits by combining the place-exchange method with other shaped nanoparticles that are more sensitive and by changing the metal to Ag.

Figure 3.20. Visible spectra of Au nanostructures on glass before and after functionalization with 0.29 µg/mL anti-IgG by the place-exchange method (5 mM) and then after subsequent exposure to 0.1 ng/mL IgG solution. $\Delta \lambda_{\text{max}}$ is 8 nm after exposure to anti-IgG and the absorbance increased by about 11.0%. After exposure to IgG, λ_{max} actually decreased by 2 nm, but the absorbance exhibited another significant increase by 9.7% to signal the presence of IgG.

3.4. CONCLUSIONS

In summary, we selectively functionalized edge and vertex sites of Au nanostructures with anti-IgG, confirming the location of thiol place-exchange on nanoparticle surfaces. We also showed experimentally that the LSPR response ($\Delta\lambda_{\text{max}}$ and $\Delta A/A_{\text{initial}}$) is significantly more sensitive to anti-IgG binding on metal nanoparticle edge and vertex sites compared to terrace sites. Finally, we used the 5 mM place-exchange anti-IgG (0.29 $\mu\text{g/mL}$) Au samples to detect IgG down to a concentration of 0.1 ng/mL, or ~ 7 pM (See Figure 3.20). This was at least 500 times lower than that detected by the pure MUA or 10% MUA samples using a 2.9 $\mu\text{g/mL}$ anti-IgG concentration and is comparable to the lowest detection limits of 100 pM and under 1 pM reported for streptavidin using Au nanorods¹⁶ and triangular Ag nanoparticles,¹⁷ respectively.

CHAPTER IV

PURIFICATION OF GOLD NANOPATES GROWN DIRECTLY ON SURFACES FOR ENHANCED LOCALIZED SURFACE PLASMON RESONANCE BIOSENSING

Here we describe the synthesis and purification of Au nanoplates grown directly on surfaces by a chemical seed-mediated growth method. The synthesis involves the attachment of 3-5 nm diameter Au nanoparticle (NP) seeds onto glass and Si/SiO_x surfaces and their subsequent growth into larger Au nanostructures by the chemical reduction of AuCl₄⁻ with ascorbic acid in the presence of cetyltrimethylammonium bromide (CTAB). We used two different growth solutions. Growth solution 1 (GS1) led to a sample with 74% Au nanospheres and 26% Au nanoplates while growth solution 2 (GS2), with lower CTAB and higher ascorbic acid concentration, led to 56% nanospheres and 44% nanoplates. The average wavelength of maximum extinction (λ_{max}) of the localized surface plasmon resonance (LSPR) band of these samples was 549 nm and 627 nm, respectively. The use of adhesive tape or sonication enables the preferential removal of spherical Au nanostructures in both cases, leaving samples with >90% Au nanoplates. The average λ_{max} increased to 672 nm (GS1) and 664 nm (GS2) for taped samples and 780 nm (GS1) and 720 nm (GS2) for sonicated samples, consistent with a higher purity of Au nanoplates on the surface. In all cases, the purified nanoplates vary in size and shape, including triangular, circular, or hexagonal structures, leading to broad spectra or

the appearance of multiple peaks. We tuned the average λ_{max} of the LSPR band of the Au nanoplate samples from 540 nm to 780 nm by varying the sonication time from 0 to 135 seconds. The change in λ_{max} upon binding of anti-IgG to the edges of the purified nanoplates increases with an increasing number of anti-IgG on the edges, is 4-8 times larger compared to that of spherical nanoparticles, and is larger for samples purified by sonication compared to taping because the former has a larger initial λ_{max} . A sample of Au nanoplates purified by taping and functionalized with anti-IgG at the edge sites displayed a shift in λ_{max} as large as 45 nm for a 10 pg/mL solution of IgG (<1 pM).

4.1. INTRODUCTION

The conduction band electrons of metal nanoparticles collectively oscillate upon interaction with electromagnetic radiation of an appropriate frequency. These oscillations are known as localized surface plasmons and this phenomenon is the basis of localized surface plasmon resonance spectroscopy (LSPR).^{68, 70} For some noble metals, especially Au and Ag, the frequency of these collective oscillations matches that of visible or near infrared light, leading to a strong absorbance or scattering in that region. The particular wavelength of maximum extinction (λ_{max}) is well-known to depend on the composition, size, and shape of the nanostructure as well as the refractive index of the surrounding environment.^{68, 70} Accordingly, many researchers have exploited the optical properties of metal nanostructures for chemical and biosensing applications. The detection of a molecular analyte of interest is possible by functionalizing a metal nanostructure with an appropriate receptor, which leads to selective binding of the molecular analyte and, if a local change in the refractive index occurs, a shift in λ_{max} of

the LSPR band. This strategy has been used previously to detect proteins,^{57, 94, 95, 101, 102, 114, 115} DNA,^{60, 81, 167} vapor molecules,⁹⁶ polymers,¹¹⁷ and metal ions.^{74, 76} The magnitude of the shift in λ_{max} depends on the number of bound analyte molecules, their distance from the metal nanostructure, the difference between the refractive index of the analyte and the global environment, the size of the analyte, the bulk refractive index sensitivity of the nanostructure, and the location of the binding analyte.¹⁵⁴

Our group has been interested in controlling and directly measuring the binding location of proteins on metal nanostructures and correlating that with the change in λ_{max} in the LSPR spectrum. In chapter III, we described the controlled attachment of human anti-IgG to the edge and vertex sites of Au nanostructures.⁴⁷ In that work, we synthesized Au nanostructures directly on surfaces that were ~77% Au nanospheres and ~23% Au nanoplates. The optical data showed that preferential binding of anti-IgG to the edge and vertex sites of these nanostructures led to dramatically higher changes in λ_{max} compared to random binding on terrace sites. For the first time, we correlated the LSPR spectra with atomic force microscopy (AFM) images that confirmed the location of binding (edges, vertices, or terraces). The main drawback of this work was that we correlated the binding location obtained from AFM images of smooth Au nanoplates with LSPR spectra dominated by the 77% Au nanospheres. While we concluded that the binding location on the Au nanoplates reflected the binding location on Au nanospheres, we still lack a direct correlation between protein binding location and the LSPR spectrum on Au nanoplates. Accordingly, our goal in this work was to synthesize samples of nearly pure Au nanoplates.

Here we describe the seed-mediated synthesis of Au nanoplates directly on glass and Si/SiO_x surfaces using two different methods with yields ranging from 26-44%, where the majority of the surface contains spherical Au nanoparticles. Importantly, we report the use of adhesive tape and sonication to selectively remove the spherical nanoparticles, resulting in surfaces with >90% Au nanoplates. Our purification strategy leads to a higher yield of nanoplates on the surface when compared to other seed-mediated growth methods directly on surfaces. To demonstrate the significance of our synthesis and purification strategy for biosensing, we attached human anti-IgG to the edge and vertex sites of the purified Au nanoplates and correlated the change in λ_{max} with the binding location and coverage as determined by AFM. Finally, we show that the λ_{max} of purified Au nanoplates on glass functionalized with human anti-IgG selectively on edge and vertex sites is highly sensitive to <1 pM IgG (10 pg/mL). Our simple benchtop chemical synthesis, functionalization, and purification method leads to highly sensitive devices for optical-based protein detection that may find use in future biomedical applications.

4.2. EXPERIMENTAL

Chemicals. Citric acid trisodium salt was purchased from Bio-rad laboratories. L-ascorbic acid (99%), sodium borohydride (98.5%), cetyltrimethyl ammonium bromide (CTAB), 11-mercaptoundecanoic acid (MUA), 2-mercaptoethanol (ME), and N-hydroxy succinimide (NHS) were purchased from Sigma-Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Pierce and 95%

mercaptopropyltrimethoxy silane (MPTMS) was purchased from Alfa Aesar. Antibody anti-human-IgG and human IgG were purchased from Sigma-Aldrich.

Synthesis of Au Nanostructures Directly on Surfaces. Au nanostructures were synthesized, purified, functionalized with anti-IgG for IgG sensing in a similar way as reported in chapter II of experimental.

4.3. RESULTS AND DISCUSSION

Synthesis of Au Nanoplates Directly on Surfaces. We synthesized Au nanostructures directly on glass and Si/SiO_x surfaces using a similar seed-mediated growth procedure described by our group previously,^{160, 162-164, 168} which is based on the solution seed-mediated synthesis of Au nanorods described by Murphy and co-workers.¹⁵⁸ In our procedure, we deposited 4 nm average diameter Au “seed” nanoparticles onto thiol-functionalized glass or Si/SiO_x substrates and then grew these seeds into larger nanostructures by electroless reduction of AuCl₄⁻ onto the seeds with ascorbic acid in the presence of cetyltrimethylammonium bromide (CTAB). Figures 4.1A and 4.1B show scanning electron microscopy (SEM) images of Si/SiO_x substrates prepared in this way using two different growth solutions. We grew the Au nanostructures in Figure 4.1A in growth solution 1 (GS1), which contained 9 mL of 0.10 M CTAB, 450 μL of 0.01 M HAuCl₄, and 50 μL of 0.1 M ascorbic acid and grew those in Figure 4.1B in growth solution 2 (GS2), which contained 9 mL of 0.016 M CTAB, 450 μL of 0.01 M HAuCl₄, and 150 μL of 0.2 M ascorbic acid. The bright features in the images correspond to individual or small aggregates of spherical Au nanoparticles and the darker circular,

Figure 4.1. Scanning electron microscopy (SEM) images of Au nanostructures grown directly on Si/SiO_x by A) growth solution 1 and B) growth solution 2. C) LSPR spectra of Au nanostructures grown on glass with growth solution 1 and 2.

hexagonal, or triangular structures are Au nanoplates as indicated in the images. We define the Au nanoplates as structures with an aspect ratio greater than 2, with the aspect ratio defined as the width of the nanoplate divided by the height. We determined the average width, height, and aspect ratio of the nanoplates using AFM or a combination of SEM (for width measurement) and AFM (for height measurement). The average width, height, and aspect ratio was 203 ± 50 nm, 37 ± 12 nm, and 6.1 ± 2.5 , respectively, based on AFM images and 163 ± 46 nm, 37 ± 12 nm, and 4.4 ± 1.9 , respectively, based on combined SEM and AFM images for nanoplates synthesized by GS1. These values were 214 ± 46 nm, 31 ± 5 nm, and 7.1 ± 2.1 , respectively, based on AFM and 184 ± 43 nm, 31 ± 5 nm, and 5.9 ± 1.7 , respectively, based on AFM and SEM, for nanoplates synthesized by GS2. The nanoplates synthesized by GS2 have a higher aspect ratio compared to those synthesized by GS1 and the aspect ratio is smaller in both cases based on the SEM measurements due to the smaller average width values, which is likely due to an exaggerated width in the AFM images due to the radius of curvature of the AFM tip. Table 4.1 shows all of the statistics of the dimensions and aspect ratio of the Au nanoplates. The yield of Au nanoplates was 26% and 44% for samples prepared with GS1 and GS2, respectively. The amount of AuCl_4^- was equal in both growth solutions, but the CTAB concentration was lower by a factor of ~ 6 and the ascorbic acid concentration was larger by a factor of 6 in GS2. It is not clear at this time why this particular change in growth solution led to a larger yield of Au nanoplates. We previously used GS1 with a different source of CTAB for growing Au nanorods directly on surfaces.^{160, 162-164, 168} With this particular CTAB source, we found a large yield of Au nanoplates on the surface. Others have recently shown that the type of Au nanostructures grown by seed-mediated growth in the presence

Table 4.1. AFM and combined AFM and SEM analysis of the average width, height, and aspect ratio of Au nanoplates synthesized by growth solutions 1 and 2 before and after tapping and sonication.

Growth solution 1							Growth solution 2					
	Before		After Taping		After Sonication		Before		After Taping		After Sonication	
	AFM	SEM + AFM	AFM	SEM + AFM	AFM	SEM + AFM	AFM	SEM + AFM	AFM	SEM + AFM	AFM	SEM + AFM
# of samples	3	3	4	3	3	3	2	3	2	3	2	3
# of Plates	46	141	43	73	41	55	16	122	23	97	18	108
Avg Length	203 ± 50	163 ± 46	183 ± 41	153 ± 33	269 ± 54	182 ± 49	214 ± 46	184 ± 43	216 ± 45	179 ± 34	202 ± 54	187 ± 48
Avg Height	37 ± 12	37 ± 12	29 ± 5	29 ± 5	28 ± 6	28 ± 6	31 ± 5	31 ± 5	32 ± 10	32 ± 10	27 ± 8	27 ± 8
Avg Aspect ratio	6.1 ± 2.5	4.4 ± 1.9	6.4 ± 2.1	5.3 ± 1.4	9.7 ± 2.6	6.5 ± 2.3	7.1 ± 2.1	5.9 ± 1.7	7.5 ± 2.8	5.6 ± 2.1	8.1 ± 2.7	6.9 ± 2.6

of CTAB is sensitive to the source of CTAB (see Experimental for more details).⁵¹ Understanding the role of the CTAB source is an important issue, but not the focus of this study. Our focus was to demonstrate a method for purifying the Au nanoplates for localized surface plasmon resonance (LSPR) sensing applications.

Figure 4.1C shows the resulting LSPR spectrum of the Au nanostructures synthesized on a glass sample using GS1 and GS2 in the seed-mediated growth method. The spectra of the Au nanostructures show an extinction maximum at about 548 nm (black) and at 631 nm (red) for those prepared using GS1 and GS2, respectively. The maximum of 548 nm is the expected position of spherical Au nanoparticles,⁴⁷ which is the dominant product (74%) in the synthesis with GS1. As the amount of Au nanoplates increased relative to the Au spherical nanoparticles using GS2 (56% spheres), the extinction maxima red-shifted to 631 nm, which is consistent with the extinction maxima of Au nanoplates reported in the literature (600-2000 nm, depending on the dimensions).^{9, 12}

Purification of Au Nanoplates by Taping. While the percentage of Au nanoplates is larger using GS2 compared to GS1, both conditions led to samples with a majority of spherical Au nanoparticles. It has been well established that the LSPR extinction maxima (λ_{max}) of nanoplates is more sensitive to refractive index changes^{109, 169} of the environment and to biomolecular binding events^{109, 169} compared to that of Au nanospheres, making it crucial to develop simple benchtop synthesis strategies for preparing substrates with a high percentage of Au nanoplates on the surface. Based on our previous success with the purification of Au and Ag nanorods (NRs),^{157, 162} we

Figure 4.2. Atomic Force Microscopy (AFM) images of Au nanoplates purified by tapping after growth on Si/SiO_x using A) growth solution 1 and B) growth solution 2. C) and D) are the corresponding LSPR spectra of samples grown on glass by growth solution 1 and 2, respectively, before (black spectrum) and after (red or blue spectrum) tapping. E) and F) show the expanded traces obtained after tapping in C) and D), respectively.

applied Scotch adhesive tape to the surface and slowly removed the tape in order to selectively remove the spherical nanoparticles. Figures 4.2A and 4.2B show atomic force microscopy (AFM) images of Si/SiO_x substrates after the synthesis of Au nanostructures using GS1 and GS2, respectively, and removal of spherical nanoparticles using the tape method. It is clear that the tape removed most of the spherical nanoparticles when comparing these images to the SEM images in Figure 4.1A and 4.1B. These samples contained $90 \pm 11 \%$ and $89 \pm 7 \%$ Au nanoplates for those prepared by GS1 and GS2, respectively, based on AFM images of at least 4 regions of 3 samples. The purified samples contained mostly Au nanoplates, although there remained a large dispersity in the particular size and shape of the nanoplate (triangular, hexagonal, circular, *etc.*). The average height, width, and aspect ratio of the Au nanoplates was 183 ± 41 nm, 29 ± 5 nm, and 6.4 ± 2.1 for those synthesized by GS1 and 216 ± 45 nm, 32 ± 10 nm, and 7.5 ± 2.8 for those synthesized by GS2, based on AFM images. These values were 153 ± 33 nm, 29 ± 5 nm, and 5.3 ± 1.4 for those synthesized by GS1 and 179 ± 34 nm, 32 ± 10 nm, and 5.6 ± 2.1 for those synthesized by GS2, based on SEM and AFM images. The average aspect ratio was similar for both synthesis methods after taping and similar to the average aspect ratio before taping in both cases based on combined SEM and AFM images. Figures 4.2C and 4.2D show the LSPR spectra before and after the taping of Au nanostructures on glass samples synthesized using GS1 and GS2, respectively. In both cases, the LSPR spectrum before taping was similar to that shown in Figure 1C, where the extinction maxima were 558 nm and 625 nm for GS1 and GS2, respectively. After taping, the extinction at all wavelengths decreased and the λ_{max} of the LSPR band red-shifted for both samples. The λ_{max} shifted from 558 nm to 672 nm and from 625 nm to

689 nm for the Au nanostructures synthesized with GS1 and GS2, respectively. Figures 4.2E and 4.2F show expanded plots of the LSPR spectra after taping. The loss in extinction after taping was due to the removal of the spherical Au nanoparticles, and likely a few nanoplates. The shift in λ_{max} occurred because the Au nanoplates, which now dominate the surface, exhibit a LSPR band between 600 and 2000 nm, depending on their exact shape, thickness, and edge length.¹² The peaks at 672 nm and 689 nm are consistent with the measured average aspect ratio of the nanoplates. The bands are fairly broad in Figures 4.2E and 4.2F, extending from 500 – 900 nm, which we believe is mainly due to the size and shape dispersity of the nanoplates. The main difference between the samples synthesized by GS1 and GS2 is that the latter had a broader spectrum and larger extinction value. The larger extinction value is consistent with the larger percentage of nanoplates on the surface prior to taping (44% versus 26%) and the broader spectrum is likely due to a larger dispersity in nanoplate size and shape. For example, based on SEM and AFM images, the deviation in aspect ratio was 38% for those synthesized by GS2 compared to 26% for those synthesized by GS1 (Table 4.1). While the λ_{max} was 67 nm more red-shifted for the sample synthesized using GS2 compared to GS1 prior to taping, after taping the λ_{max} value was similar (difference of 17 nm), consistent with a high purity of Au nanoplates for both samples.

Purification of Au Nanoplates by Sonication. We also purified samples of Au nanostructures by subjecting the glass or Si/SiO_x slides to sonication. Figures 4.3A and 4.3B show AFM images of Si/SiO_x substrates after the synthesis of Au nanostructures using GS1 and GS2, respectively, and removal of spherical nanoparticles by sonication in

water. As with the tape method, it is clear that sonication removed most of the spherical nanoparticles when comparing these images to the SEM images in Figure 4.1. These samples contained $91 \pm 9 \%$ and $90 \pm 8 \%$ Au nanoplates for those prepared by GS1 and GS2, respectively, based on AFM images of at least 5 regions of 3 samples. The average height, width, and aspect ratio of the Au nanoplates was 269 ± 54 nm, 28 ± 6 nm, and 9.7 ± 2.6 for those synthesized by GS1 and 202 ± 54 nm, 27 ± 8 nm, and 8.1 ± 2.7 for those synthesized by GS2, based on AFM images. These values were 182 ± 49 nm, 28 ± 6 nm, and 6.5 ± 2.3 for those synthesized by GS1 and 187 ± 48 nm, 27 ± 8 nm, and 6.9 ± 2.6 for those synthesized by GS2, based on SEM and AFM images. The average aspect ratio was slightly larger for the samples synthesized by GS1 compared to GS2 after sonication based on the AFM images, but similar for both methods based on the combined SEM and AFM images.

Figures 4.3C and 4.3D show the LSPR spectra before and after sonication of Au nanostructures on glass samples synthesized using GS1 and GS2, respectively. In both cases, the LSPR spectrum before sonication was again similar to that shown in Figure 4.1C and Figure 4.2, where the extinction maxima was 548 nm and 617 nm for GS1 and GS2, respectively. After sonication, the extinction at all wavelengths decreased and the λ_{max} of the LSPR band red-shifted to 789 nm and 717 nm for the Au nanostructures synthesized with GS1 and GS2, respectively, consistent with their measured average aspect ratios. Figures 4.3E and 4.3F show expanded plots of the LSPR spectra after sonication. Similar to taped samples, the extinction decreased and λ_{max} red-shifted

Figure 4.3. Atomic Force Microscopy (AFM) images of Au nanoplates purified by sonication after growth on Si/SiO_x using A) growth solution 1 and B) growth solution 2. C) and D) are the corresponding LSPR spectra of samples grown on glass by growth solution 1 and 2, respectively, before (black spectrum) and after (red or blue spectrum) sonication. E) and F) show the expanded plots after sonication in C) and D), respectively.

significantly, indicative of a sample with a high percentage of nanoplates. The bands were fairly broad, often displaying multiple peaks due to the size and shape dispersity of the nanoplates. The sample shown in Figure 4.3E, for example, displayed a main LSPR peak at 789 nm, but there were also two shoulder peaks at about 600 nm and 700 nm. The LSPR spectrum of the sample shown in Figure 4.3F displayed a main peak at 717 nm and two shoulders at 640 nm and at 850 nm that are likely due to other nanoplate populations present on the substrate. See Figure 4.4 for examples of other sonicated samples with multiple peaks in their LSPR spectrum.

We synthesized several samples using GS1 and GS2 and purified them by taping or sonication. Table 4.2 shows the average $\lambda_{\max}$ values and standard deviation of the major peak from all of the samples prepared ($n = 3$ to 7 samples). The average $\lambda_{\max}$ followed the order of GS1 (549 ± 6 nm) < GS2 (627 ± 13 nm) < GS2 Taped (664 ± 23 nm) $\approx$ GS1 Taped (672 ± 15 nm) < GS2 Sonicated (720 ± 12 nm) < GS1 Sonicated (780 ± 47 nm). The taped samples synthesized by GS1 and GS2 are statistically the same and the $\lambda_{\max}$ is less than the sonicated samples. The GS1 sonicated sample has a slightly larger $\lambda_{\max}$ value compared to the GS2 sonicated sample.

Table 4.2 provides the average extinction at $\lambda_{\max}$ with standard deviation for samples synthesized by GS1 and GS2 and taped or sonicated. As expected and discussed earlier, the average extinction of the taped GS2 sample was ~ 3 times larger compared to the taped GS1 sample (0.068 ± 0.014 versus 0.027 ± 0.022). This was due to the higher percentage of nanoplates on the GS2 sample before taping compared to the GS1 sample (44% versus 26%). In contrast, the extinction value of the GS1 sample was larger compared to GS2 after sonication (0.024 ± 0.008 versus 0.011 ± 0.005). This shows that

Figure 4.4. LSPR spectra of Au nanostructures synthesized by seed-mediated growth and then sonicated in water for 5 min. The λ_{max} values are more red-shifted compared to taped samples and the spectra often exhibit multiple peaks, which we attribute to different nanoplate populations.

Table 4.2. Data table showing the % of nanoplates, average wavelength, average extinction and average aspect ratio of Au nanoplates synthesized and purified by the different methods.

Synthesis Strategy		% Au Nanoplates	$\lambda_{\max}$ (nm)	Extinction at $\lambda_{\max}$	Aspect Ratio	
					AFM Only	SEM + AFM
Growth solution 1	As-Prepared	23 ± 7	549 ± 6	0.121 ± 0.025	6.1 ± 2.5	4.4 ± 1.9
	Taped	90 ± 11	672 ± 15	0.027 ± 0.022	6.4 ± 2.1	5.3 ± 1.4
	Sonicated ^a	91 ± 9	780 ± 47	0.024 ± 0.008	9.7 ± 2.6	6.5 ± 2.3
Growth solution 2	As-Prepared	44 ± 8	627 ± 13	0.134 ± 0.019	7.1 ± 2.1	5.9 ± 1.7
	Taped	89 ± 7	664 ± 23	0.068 ± 0.014	7.5 ± 2.8	5.6 ± 2.1
	Sonicated ^b	90 ± 8	720 ± 12	0.011 ± 0.005	8.1 ± 2.7	6.9 ± 2.6

^a Sonication time was 5 minutes.

^b Sonication time was 2-3 minutes. Longer times often led to more significant removal of the Au nanostructures.

sonication removed many of the Au nanoplates from the surface in addition to the spherical particles and that nanoplates synthesized by GS2 were easier to remove compared to those synthesized by GS1, which is not well understood. Tables 4.3 - 4.6 show the λ_{max} and extinction values of all samples synthesized by GS1 and GS2 and purified by tape or sonication.

Table 4.2 also shows the average aspect ratio determined by AFM only or the combination of SEM and AFM (to measure the average width and height, respectively) of samples synthesized and purified by the different methods. The average aspect ratio of GS1 as prepared, taped and sonicated were 6.1 ± 2.5 , 6.4 ± 2.1 , and 9.7 ± 2.6 as determined by AFM and 4.4 ± 1.9 , 5.3 ± 1.4 , and 6.5 ± 2.3 as determined by SEM and AFM, respectively, whereas the average aspect ratio values of GS2 as prepared, taped and sonicated were 7.1 ± 2.1 , 7.5 ± 2.8 , and 8.1 ± 2.7 as determined by AFM and 5.9 ± 1.7 , 5.6 ± 2.1 , and 6.9 ± 2.6 as determined by SEM and AFM, respectively. In general, the average λ_{max} values in Table 4.2 increase with an increase in the % of nanoplates and increase in the average aspect ratio. For example, as-prepared nanoplate samples prepared by GS2 have a higher % of nanoplates, higher average aspect ratio, and a larger average λ_{max} compared to samples prepared by GS1. Taped samples have a larger % of nanoplates, a larger or similar aspect ratio, and a larger average λ_{max} compared to the as-prepared samples. Finally, sonicated samples have a larger average aspect ratio and larger average λ_{max} compared to taped samples.

Table 4.3. The wavelength of maximum extinction ($\lambda_{\max}$) and extinction of Au nanostructures synthesized by growth solution 1 before and after purification by tape.

Growth solution 1						
Sample	Before taping		After taping			
			Peak 1		Peak 2	
	$\lambda_{\max}$	Ext	$\lambda_{\max}$	Ext	$\lambda_{\max}$	Ext
1	548	0.139	672	0.020	888	0.016
2	541	0.128	678	0.016	778	0.015
3	558	0.096	647	0.064		
4	550	0.083	674	0.009		
5	554	0.139	694	0.009		
6	543	0.140	668	0.045		
7						
Avg	549	0.121	672	0.027	833	0.016
Std. Dev.	6	0.025	15	0.022	77	0.001

Table 4.4. The wavelength of maximum extinction ($\lambda_{\max}$) and extinction of Au nanostructures synthesized by growth solution 1 before and after purification by sonication.

Growth solution 1						
Sample	Before sonication		After sonication			
			Peak 1		Peak 2	
	$\lambda_{\max}$	Ext	$\lambda_{\max}$	Ext	$\lambda_{\max}$	Ext
1	548	0.114	789	0.035		
2	570	0.154	858	0.035		
3	542	0.113	739	0.020		
4	551	0.112	792	0.013	882	0.012
5	539	0.142	725	0.023		
6	543	0.129	814	0.026		
7	547	0.116	746	0.017	880	0.016
Avg	548	0.126	780	0.024	881	0.014
Std. Dev.	10	0.017	47	0.008	1	0.003

Table 4.5. The wavelength of maximum extinction (λ_{max}) and extinction of Au nanostructures synthesized by growth solution 2 before and after purification by tape.

Growth solution 2						
Sample	Before taping		After taping			
			Peak 1		Peak 2	
	λ_{max}	Ext	λ_{max}	Ext	λ_{max}	Ext
1	615	0.155	643	0.082		
2	625	0.119	689	0.054		
3	641	0.128	660	0.068		
Avg	627	0.134	664	0.068		
Std. Dev.	13	0.019	23	0.014		

Table 4.6. The wavelength of maximum extinction ($\lambda_{\max}$) and extinction of Au nanostructures synthesized by growth solution 2 before and after purification by sonication.

Growth solution 2						
Sample	Before sonication		After sonication			
			Peak 1		Peak 2	
	$\lambda_{\max}$	Ext	$\lambda_{\max}$	Ext	$\lambda_{\max}$	Ext
1	607	0.168	701	0.018		
2	620	0.149	717	0.012		
3	627	0.157	722	0.012		
4	637	0.110	739	0.011		
5	643	0.131	722	0.012		
6	635	0.129	722	0.003	840	0.003
Avg	628	0.141	720	0.011		
Std. Dev.	13	0.021	12	0.005		

Tuning the LSPR Extinction Maxima of Au Nanoplates with Sonication Time. We obtained the LSPR spectra in Figure 4.3 after sonicating the samples synthesized by GS1 and GS2 for 5 min and 2 min, respectively. In order to better understand the details of the removal of the spherical nanoparticles, we monitored the LSPR spectra as a function of sonication time. Figures 4.5A and 4.5B show the LSPR spectra of Au nanostructures synthesized on glass using GS1 and GS2, respectively, before and after sonication for various times ranging from 0 to 135 s and 0 to 25s. The nanostructures synthesized with GS1 (Figure 4.5A) had an initial LSPR λ_{max} of 539 nm at 0 s, which increased with increasing sonication time until a final value of 728 nm after 135 s. We normalized the extinction values to the initial extinction value at 0 s in Figures 4.5A and 4.5B in order to clearly show the shift in λ_{max} and details of the LSPR peak with sonication time. The extinction actually decreased by a factor of 7-8 following sonication after the 135 s due to the loss of Au nanospheres and some nanoplates from the surface. Figure 4B shows the results of a similar experiment performed on Au nanostructures synthesized on glass with GS2. In this case, λ_{max} was 648 nm at 0 s and gradually shifted to 704 nm after 15 s and 723 nm after 25 s of sonication. Note that while the major peak of the final spectrum was 723 nm, there was a second large peak around 780 nm and even a third peak near 900 nm. We used shorter sonication times for Au nanostructures synthesized by GS2 because in many cases, a longer sonication time removed all of the Au from the surfaces as mentioned earlier. Figures 4.5C and 4.5D show plots of λ_{max} (left axis) and extinction (right axis) as a function of sonication time for Au nanostructures synthesized on glass using GS1 and GS2, respectively. The general trend consistently showed an increase in λ_{max} and decrease in extinction with increasing sonication time for both growth solutions,

Figure 4.5. Normalized LSPR spectra of Au nanostructures synthesized by A) growth solution 1 and B) growth solution 2 before and after sonication for the indicated times. All spectra were normalized to the absorbance of the spectrum at 0 s. The inset shows a zoom-in of the major LSPR bands with time and the λ_{max} values. Plots of wavelength of maximum extinction (λ_{max}) on the left y-axis (red) and extinction at λ_{max} on the right y-axis (black) as a function of sonication time for Au nanostructures synthesized by C) growth solution 1 and D) growth solution 2. The black and red lines were hand drawn as a guide to show the general trends.

although the exact values with time have variability. The extinction decreased with sonication time due to the removal of spherical nanoparticles and some Au nanoplates from the surface. The increase in λ_{max} was due to a higher population of nanoplates on the surface. Previous results showed that λ_{max} increases for Au nanoplates as the aspect ratio increases.¹² Considering this fact, our results suggest that with increasing sonication time, the average aspect ratio of the Au nanoplates remaining on the surface increased.

In order to determine if the average aspect ratio of the Au nanoplates increased with increasing sonication time, we obtained AFM images of the Au nanoplates at different sonication times. Figure 4.6 shows AFM images of Au nanostructures synthesized on a glass surface using GS1 before sonication (Frame A), after sonication for 15 s (Frame B), and after sonication for 55 s (Frame C). As shown in Figure 4.1, the surface initially contained a large population of spherical nanoparticles before sonication in Figure 4.6A. An analysis of the aspect ratio of at least 15 different Au nanoplates showed an average of 5.9 ± 2.4 . The initial λ_{max} of this sample was 540 nm. The average aspect ratio and λ_{max} were 5.8 ± 2.4 and 656 nm, respectively, after 15 s of sonication (Frame B). Since the average aspect ratio did not change significantly, the red-shift in λ_{max} was due to the higher percentage of Au nanoplates upon removal of the spherical nanoparticles as opposed to a change in the average aspect ratio of the Au nanoplates. The average aspect ratio and λ_{max} was 10.2 ± 2.8 and 734 nm, respectively, after 55 s of sonication (Frame C). In this case, the red-shift in λ_{max} was due to further purification of the Au nanoplates, but also due to the increase in the average aspect ratio of the Au nanoplates that remained on the surface.¹² The AFM determined average aspect ratio (see Table 4.7) and corresponding λ_{max} are close to the calculated extinction spectra

Figure 4.6. AFM images of Au nanostructures synthesized by growth solution 1 before sonication (A) and after sonication for 15 s (B) and 55 s (C). The images show the width of the major axis of one nanoplatform and the inset shows the height analysis of the same nanoplatform along with the calculated aspect ratio of that nanoplatform. The aspect ratio of the nanoplatform shown is close to the average aspect ratio of the sample based on measurements from at least 15 nanoplatforms as also shown in the inset. The aspect ratio is the width of the major axis divided by the nanoplatform height.

Table 4.7. AFM analysis of the aspect ratio of Au nanostructures synthesized by growth solution 1 before and after sonication for various times.

Growth solution 1										
Sample	1			2			3			
Sonication time (secs)	0	15	55	0	15	55	0	20	55	105
Average Aspect ratio	5.9 ± 2.4	5.8 ± 2.4	10.2 ± 2.8	5.6 ± 2.0	6.1 ± 2.6	9.3 ± 2.0	6.9 ± 3.0	5.8 ± 2.5	9.1 ± 2.6	9.6 ± 2.9
Average length	207 ± 40	205 ± 43	277 ± 47	192 ± 39	191 ± 75	287 ± 57	214 ± 70	210 ± 43	247 ± 71	246 ± 54
Average height	39 ± 14	38 ± 9	28 ± 6	38 ± 13	32 ± 7	31 ± 6	33 ± 8	42 ± 16	27 ± 5	27 ± 6

reported by Tominaga and co-workers² and the experimental results reported by Yun and co-workers.¹² Based on the AFM and LSPR data, we conclude that sonication removes spherical nanoparticles first followed by low aspect ratio nanoplates that are relatively smaller in width and larger in height. Wider and thinner plates ultimately survive the sonication and remain on the surface. These nanoplates have a larger aspect ratio with λ_{max} values at longer wavelengths. We believe that the high aspect ratio Au nanoplates adhere best to the surface because they have a larger contact area with the surface compared to low aspect ratio nanoplates or spherical nanoparticles (aspect ratio ~ 1). At this point, we do not have any evidence that the sonication procedure directly alters the size and shape of the nanoplates, although this is a possibility due to the heat generated.³⁰

LSPR Response of Au Nanoplates to Anti-IgG Binding. We used our previously published method to attach anti-IgG selectively to the edge sites of the Au nanoplates synthesized using GS1 and GS2 and purified by taping and sonication.⁴⁷ With this procedure, we attached human anti-IgG from a 0.26 $\mu\text{g/mL}$ solution onto the Au nanoplates by an amide coupling reaction to the carboxylic acid groups of mercaptoundecanoic acid (MUA), which was place-exchanged from a 5 mM or 6 mM solution onto Au nanoplates functionalized first with mercaptoethanol (ME) (see Experimental for full details).⁴⁷ We measured the collective LSPR spectrum of the Au nanoplates from the same exact location on carefully-marked glass substrates before and after attachment of anti-IgG. Figure 4.7 shows the LSPR spectra of glass samples containing Au nanoplates synthesized by GS1 and purified by the tape procedure (Frame A and Frame B) or by sonication (Frame C and Frame D) before and after anti-IgG

Figure 4.7. LSPR spectra of Au nanoplates synthesized by growth solution 1 and purified by (A, B) tapping and (C, D) sonication before (black) and after (red) functionalization with 0.26 $\mu\text{g/mL}$ human anti-IgG using (A, C) 5 mM and (B, D) 6 mM place-exchanged MUA. AFM images of Au nanoplates purified by tapping and functionalized with 0.26 $\mu\text{g/mL}$ human anti-IgG using E) 5 mM and F) 6 mM place-exchanged MUA.

functionalization using a 5 mM (Frame A and Frame C) and 6 mM (Frame B and Frame D) place-exchanged MUA linker. We previously showed that attachment of anti-IgG to MUA that was place-exchanged from a 5 mM and a 6 mM solution led to a greater amount of anti-IgG on the edge sites for the latter.⁴⁷ The LSPR spectra of nanoplates purified by taping in Figures 4.7A and 4.7B show a shift in λ_{max} of 22 nm and 35 nm for attachment of anti-IgG to 5 mM and 6 mM exchanged MUA, respectively, while those of nanoplates purified by sonication in Figures 4.7C and 4.7D show a shift in λ_{max} of 42 nm and 47-68 nm for attachment of anti-IgG to 5 mM and 6 mM exchanged MUA, respectively. In three of the four cases the extinction also increased, but we showed previously that this is less reproducible than the shift in λ_{max} .⁴⁷ In both cases, the larger shift for the 6 mM MUA samples shows that more anti-IgG attached to the edges of the nanoplates compared to the 5 mM samples. The sonicated samples showed a larger shift in λ_{max} upon anti-IgG binding compared to taped samples due to the larger initial λ_{max} value for these nanostructures, as it has been shown that the sensitivity to a refractive index change in the environment increases with increasing initial λ_{max} .^{109, 148, 149, 170} This is also evident from the fact that peak 2 in Figure 4.7D was more sensitive to anti-IgG binding compared to peak 1 in the same spectrum. Control experiments with pure ME treated samples showed $\Delta\lambda_{\text{max}}$ of only 1 nm (see Figure 4.8) when treated with anti-IgG. We repeated this important control experiment several times with similar results, which confirms that 1) human anti-IgG covalently binds to the MUA linker and 2) that we were successful at monitoring the same location of the sample before and after functionalization. Figure 4.7E and 4.7F shows AFM images of two Au nanoplates synthesized on Si/SiO_x using GS1, purified by taping, and then functionalized with

Figure 4.8. LSPR spectra of a taped sample of Au nanoplates functionalized with mercaptoethanol (ME) and exposed to 0.26 µg/mL human anti-IgG. The small shift in λ_{max} indicates little or no anti-IgG binding on the surface and the nearly identical LSPR spectrum before and after anti-IgG exposure confirms that we were able to successfully obtain the spectrum in the same region of the sample before and after anti-IgG exposure.

anti-IgG by coupling to 5 mM and 6 mM place-exchanged MUA, respectively. The darkest regions correspond to the underlying Si/SiO_x substrate, the intermediate shade correspond to the triangular or circular Au nanoplates, and the bright regions on top of the nanoplates correspond to the anti-IgG. The very big bright spot in Figure 4.7F is a large spherical nanoparticle that was not removed during taping. The AFM images confirm that anti-IgG attached preferentially to the edges of the Au nanoplates and that the coverage increased with increasing MUA concentration as shown previously for non-purified nanoplate samples.⁴⁷ The AFM is consistent with the fact that the $\lambda_{\max}$ shift was largest for the anti-IgG attached to 6 mM place-exchanged MUA.

Table 4.8 shows the average initial $\lambda_{\max}$ ($\lambda_{\max, \text{init}}$) and shift in $\lambda_{\max}$ ($\Delta\lambda_{\max}$) of the Au nanostructures synthesized by GS1 and GS2 before (as-prepared) and after purification by tape and sonication for the nanostructures synthesized by GS1. For nanostructures synthesized by GS1 and functionalized with human anti-IgG through 5 mM place-exchanged MUA, the $\Delta\lambda_{\max}$ increased in the order of as-prepared (6 ± 3 nm) < taped (23 ± 1 nm) < sonicated (49 ± 13 nm). Based on the data in Tables 4.2 and 4.8, the $\Delta\lambda_{\max}$ increases with increasing percentage of Au nanoplates and increasing initial $\lambda_{\max, \text{init}}$ of the nanoplates. The as-prepared sample had 26% Au nanoplates on the sample and the lowest $\lambda_{\max, \text{init}}$ (547 nm) while the sonicated sample had ~90% Au nanoplates and largest $\lambda_{\max, \text{init}}$ of 766 nm. The $\Delta\lambda_{\max}$ also increased with increasing percentage of Au nanoplates and $\lambda_{\max, \text{init}}$ for samples functionalized by human anti-IgG through 6 mM place-exchanged MUA. The $\Delta\lambda_{\max}$ was about 50% larger when compared to the 5 mM place-

Table 4.8. Initial $\lambda_{\max}$ ($\lambda_{\max, \text{init}}$) and response to human anti-IgG binding of the Au nanoplate samples synthesized, purified, and functionalized by different methods.

Synthesis Strategy		5 mM MUA		6 mM MUA	
		$\lambda_{\max, \text{init}}$ (nm)	$\Delta\lambda_{\max}$ (nm)	$\lambda_{\max, \text{init}}$ (nm)	$\Delta\lambda_{\max}$ (nm)
Growth Solution 1	As-Prepared	547 ± 12	6 ± 3	545 ± 4	9 ± 1
	Taped	644 ± 52	23 ± 1	675 ± 54	32 ± 3
	Sonicated	766 ± 70	49 ± 13	704 ± 8	53 ± 4
Growth Solution 2	As-Prepared	624 ± 9	16 ± 2	642 ± 36	22 ± 3

exchanged samples in all cases except for those synthesized by GS1 and sonicated. The general increase in $\Delta\lambda_{\max}$ for the 6 mM samples occurred because of the larger number of anti-IgG on the nanoplate surface as determined by AFM. The smaller difference between 6 mM and 5 mM in the case of the sonicated samples synthesized by GS1 could be due to the larger $\lambda_{\max,\text{init}}$ for the 5 mM samples (by 62 nm), which makes those nanoplates more sensitive to refractive index changes. The magnitude of $\Delta\lambda_{\max}$ for the as-prepared GS2 samples lies between the as-prepared GS1 samples and taped GS1 samples, consistent with the percentage of Au nanoplates and $\lambda_{\max,\text{init}}$ for this sample shown in Tables 4.2 and 4.6. Importantly, our results show that samples of purified nanoplates, by sonication or taping, exhibit a significantly larger LSPR response to protein binding compared to the as-prepared samples containing mostly spherical nanoparticles.

Sensing IgG.

We performed a preliminary test on the use of surface-attached Au nanoplates synthesized by GS1, purified by tape, and functionalized with human anti-IgG for the detection of human IgG. Figure 4.9 shows the LSPR spectra of the Au nanoplates before (black spectrum) and after (red spectrum) edge-functionalization with human anti-IgG using 6 mM place-exchanged MUA as the linker. Attachment of anti-IgG led to a shift from 624 nm to 657 nm ($\Delta\lambda_{\max} = 33$ nm) and 763 nm to 800 nm ($\Delta\lambda_{\max} = 37$ nm) for the first and second LSPR peaks, respectively. These shifts are consistent with those observed for other taped samples synthesized by GS1 as shown in Figure 4.7 and Table 4.2. Subsequent exposure to a 10 pg/mL solution of IgG led to a further shift from 657

Figure 4.9. LSPR spectra showing a glass sample coated with tape purified Au nanostructures synthesized by growth solution 1 before functionalization with human anti-IgG (black), after functionalization with human anti-IgG using 6 mM place-exchanged MUA (red), and after exposure to 10 pg/mL human IgG (green).

nm to 662 nm ($\Delta\lambda_{\text{max}} = 7$ nm) and 800 nm to 845 nm ($\Delta\lambda_{\text{max}} = 45$ nm) for the first and second LSPR peaks (green spectrum), respectively. Interestingly, the extinction value increased dramatically for the first peak and slightly decreased for the second peak while the second peak exhibited a much larger shift in λ_{max} . These two different populations of Au nanoplates on the same sample (peak 1 and 2) showed similar results upon anti-IgG binding, but drastically different responses to IgG. The reason is not clear at this time. This interesting phenomenon can only be elucidated by correlating LSPR spectroscopy data with AFM imaging of the protein binding location at the single nanoplate level. This is an exciting topic that we will explore in the future. Importantly, this sample exhibited a much larger $\Delta\lambda_{\text{max}}$ in response to a ten times lower IgG concentration (10 pg/mL or < 1 pM) compared to our previous work on samples containing a majority of Au nanospheres.⁴⁷ This is among the lowest concentration detected by LSPR for proteins to date.¹⁷¹ The detailed aspects of human IgG sensing with purified Au nanoplates, including kinetics, sensitivity, and limit of detection will be described separately.

Refractive Index Sensitivity.

We performed refractive index sensitivity studies on a non-purified sample synthesized by GS1 (containing mostly spherical nanoparticles) and on three samples synthesized by GS1 and purified by sonication. Figure 4.10 shows the LSPR spectra obtained in air, water, 2-propanol, toluene and then back in air. The λ_{max} of the LSPR band increased with increasing refractive index (RI) of the solvent as expected. Also, the

Figure 4.10. LSPR spectra of samples synthesized by GS1 and (A) not purified or (B-D) purified by sonication. We exposed the sample to solvents of increased refractive index following the order air, water, 2-propanol, toluene and back in air, respectively.

spectrum in air before exposure to the solvents matches the spectrum after exposure to the different solvents, indicating that the nanoplate samples were stable and the spectra were from the same region of the glass sample. Importantly, we found that the extinction intensity increased in some cases as we also observed for anti-IgG or IgG binding to the Au nanoplates. Figure 4.11 shows plots of the change in λ_{max} as a function of the RI of the solvent. From the slope, the RI sensitivity was 89 nm/RIU for the non-purified sample with a λ_{max} of 539 nm and 195 and 302 nm/RIU for the purified nanoplate samples with a λ_{max} of 677 and 828 nm, respectively. As expected, the RI sensitivity increased as the initial λ_{max} of the LSPR band increased. In addition, toluene has a RI closest to that expected for proteins such as anti-IgG, therefore the 90-150 nm shift, depending on the λ_{max} , represents the upper limit of the λ_{max} shift for a full coverage of anti-IgG on the nanoplate surface. The fact that we observe shifts of 50 nm in some cases for anti-IgG bound to nanoplate edge sites, which is one-third to one-half of the maximum signal, shows that the edge sites are highly sensitive since the nanoplate surface is not nearly one-third or one-half covered by the anti-IgG based on the AFM images.

4.4. CONCLUSIONS

We demonstrated the seed-mediated synthesis of Au nanoplates directly on surfaces using two different growth solutions and selectively removed Au nanospheres by tape or sonication to form samples with ~90% Au nanoplates. We believe that the smooth, flat morphology of the Au nanoplates provides a larger contact area with the surface that allows them to adhere more strongly compared to Au spheres. The different synthesis and purification strategies led to different λ_{max} values for the Au nanoplate samples,

Figure 4.11. Plot of refractive index vs wavelength shift for spherical Au particles with a $\lambda_{\max}$ of 539 nm and nanoplates with a $\lambda_{\max}$ of 677 nm and 828 nm as indicated.

ranging from 549 nm up to 780 nm, and the LSPR λ_{max} could be tuned by altering the sonication time. The λ_{max} value increases with increasing purity of the nanoplates and increasing aspect ratio of the plates that remain on the surface. The λ_{max} of purified nanoplates from the LSPR spectrum after edge-functionalization with human anti-IgG shifted by 22 nm up to 68 nm, which is 4-8 times larger compared to samples dominated by Au nanospheres, depending on the amount of anti-IgG attached and the purification strategy. Au nanoplates synthesized by GS1 and sonicated for 5 minutes had the largest initial λ_{max} and largest shift upon anti-IgG binding. In addition, a tape purified Au nanoplate sample edge-functionalized with anti-IgG detected 10 pg/mL of IgG with a large 45 nm shift for the largest λ_{max} LSPR band. This shows that these purified Au nanoplates have great promise for use in highly sensitive protein detection. This work is important because our synthesis and purification strategy involves simple benchtop procedures that do not require high vacuum evaporation or sputtering, lithography, or other complex or expensive methods. Because the growth is directly on surfaces, we eliminate the need for assembling Au nanoplates onto a surface from solution. The protein functionalization strategy importantly shows that the LSPR spectrum of Au nanoplates is highly sensitive to the binding of just a few protein molecules on the edge, which may also be useful for monitoring molecular binding to metal nanostructure edge sites by surface-enhanced Raman spectroscopy (SERS).

CHAPTER V

THE EFFECT OF PROTEIN BINDING LOCATION AND DISTANCE ON THE LOCALIZED SURFACE PLASMON RESONANCE RESPONSE OF PURIFIED AU NANOPATES GROWN DIRECTLY ON SURFACES

Here we report the effect of protein binding location and binding distance on the localized surface plasmon resonance (LSPR) response of purified Au nanoplates grown directly on surfaces. The response to binding of anti-IgG on the nanoplate surface depends strongly on both variables. We experimentally controlled the binding location between nanoplate terrace and edge sites by the method of anti-IgG attachment and the binding distance by altering the chain length of the linker that the anti-IgG binds to. The LSPR response to anti-IgG binding increases with increasing initial $\lambda_{\max}$ and depends dramatically on the anti-IgG binding location, which we control experimentally. The average change in $\lambda_{\max}$ ($\Delta\lambda_{\max}$) for binding to terraces at medium coverage, edge sites at low coverage and edge sites at medium coverage using pure MUA, 5 mM and 6 mM place-exchange MUA are 21 ± 5 nm, 49 ± 13 nm and 53 ± 4 nm on samples purified by sonication, 12 ± 1 nm, 23 ± 1 nm and 32 ± 3 nm on samples purified by adhesive tape, and 1 ± 1 nm, 6 ± 3 nm and 9 ± 1 nm on non purified samples. Atomic force microscopy (AFM) images reveal the binding location of anti-IgG and, when correlated to the UV-vis spectroscopy of the nanoplates, confirms that the edge sites are more sensitive to protein

binding. This leads to the estimated detection of only a few anti-IgG molecules on one nanoplate due to a high localized electric field enhancement and small effective sensing volume at the edge sites. The LSPR response increases as the chain length of the linker decreases. The LSPR shift increases in the order of mercaptopropionicacid (MPA) > 11-mercaptopundecanoicacid (MUA) > 16-mercaptohexadecanoicacid (MHDA) as linkers for anti-IgG. The average changes in $\lambda_{\max}$ ($\Delta\lambda_{\max}$) for 5 mM MPA, MUA and MHDA are 47 ± 2 nm, 49 ± 13 nm and 33 ± 2 nm on sonicated samples and 33 ± 1 nm, 23 ± 1 nm and 20 ± 1 nm for taped samples and 7 ± 1 nm, 6 ± 3 nm and 3 ± 0 nm for non-purified samples while those for 6 mM exchange are 61 ± 5 nm, 53 ± 4 nm and 42 ± 3 nm for sonicated samples and 39 ± 3 nm, 32 ± 3 nm, 25 ± 1 nm for taped samples and 12 ± 2 nm, 9 ± 1 nm and 6 ± 1 nm for non-purified samples. We further compare the IgG response on the purified nanoplates where sample with edge-attached anti-IgG showed a shifts of 9 nm for 0.01 ng/mL as opposed to those with terrace bound anti-IgG which showed shifts of 7 nm for 1 ng/mL. This work is important for understanding and optimizing the LSPR response to protein binding for bioanalytical applications.

5.1. INTRODUCTION.

The following equation describes the change in the wavelength of maximum extinction ($\Delta\lambda_{\max}$) when a molecule binds to the nanostructure:¹³⁸

$$\Delta\lambda_{\max} = [3 \cdot S_0 \cdot \exp(-2r/l_d)/V_s][\Delta RI \cdot N \cdot V_A] \quad (5.1)$$

where V_s is the sensing volume.¹³⁸ ΔRI is the refractive index (RI) difference between the medium and the binding molecule, N is the number of molecules bound, V_A is the volume of one individual analyte molecule, S_0 is the bulk sensitivity and l_d is the decay length of Plasmon. $\Delta\lambda_{\max}$ decreases exponentially as r increases due to the exponential decrease in the electromagnetic field enhancement with distance from the nanoparticle surface.

We previously showed the controlled binding of anti-IgG to the edge and vertex sites of spherical Au nanoparticles and Au nanoplates synthesized directly on glass and Si/SiO_x surfaces. Atomic force microscopy (AFM) confirmed that anti-IgG preferentially attached to edge and vertex sites of Au nanoplates using place-exchanged self-assembled monolayers (SAMs) as linkers. LSPR spectra showed that these samples exhibited significantly larger $\Delta\lambda_{\max}$ and larger absorbance increases compared to samples with anti-IgG randomly bound to the flat terrace regions. This was true even when the coverage of anti-IgG was much lower on the former. One problem in our previous work was that we obtained AFM images from individual Au nanoplates to determine binding location, but monitored the collective optical properties of the entire sample, which was dominated by

spherical Au nanoparticles. The AFM data therefore did not directly correlate with the UV-vis spectroscopy data. In this chapter, we describe the LSPR response as a function of protein binding location and distance on purified Au nanoplates samples with > 90% nanoplates. This allowed us to directly correlate the collective optical properties of purified Au nanoplates with AFM images showing the binding location of anti-IgG on the Au nanoplates. We also report the effect of binding distance by exchanging with SAMs of different carbon chain lengths and noticed that the LSPR response is more pronounced when proteins are bound close to the surface than farther away from the surface. This work is important as it will impact the use of metal nanostructures for LSPR- and SERS-based chemical and biochemical sensing.

5.2. EXPERIMENTAL

Chemicals

Citric acid trisodium salt was purchased from Bio-rad laboratories. L-ascorbic acid (99%), sodiumborohydride (98.5%), cetyl trimethyl ammonium bromide (CTAB), 11-mercaptopundecanoic acid (MUA), 2-mercapto ethanol (ME), N-hydroxy succinimide (NHS) were purchased from Sigma-Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Pierce and Merapto propyl trimethoxy silane, 95% (MPTMS) was purchased from Alfa Aesar. Antibody anti-human-IgG and antigen human-IgG were purchased from Sigma-Aldrich.

Synthesis of Au Nanostructures Directly on Surfaces. We synthesized Au nanostructures on glass and Si/SiO_x surfaces, purified, functionalized with anti-IgG for IgG detection in a similar way as discussed in Chapter II.

5.3. RESULTS AND DISCUSSION

Effect of Binding Location on LSPR Response. We functionalized the purified Au nanoplates with antihuman-IgG by two methods as described previously.⁴⁷ Figure 5.1 shows an AFM image of a sample purified by adhesive tape after synthesis by GS1 and the inset shows a zoom-in of a typical nanoplate (Frame A) and anti-IgG immobilization using pure MUA (Frame B), 5 mM MUA place-exchange (Frame C) and 6 mM MUA place-exchange (Frame D). The AFM shows selective attachment of anti-IgG to the edges when exchanged with 5 or 6 mM MUA where as pure MUA method has anti-IgG immobilized randomly on the terraces.

Figure 5.2 shows the LSPR spectra of glass samples containing Au nanoplates purified by the ultrasonication procedure before and after anti-IgG functionalization (0.26 µg/mL) by the pure MUA (Frame A), 5 mM place-exchange (Frame B), and 6 mM place-exchange (Frame C) strategies. Frame D shows the result of a pure ME sample before and after human anti-IgG exposure as a control. All of the samples displayed a LSPR band between 600 and 800 nm before functionalization as reported by our group previously.¹⁷² Anti-IgG attachment by the pure MUA method led to a red-shift in the LSPR band to longer wavelengths by 25 nm ($\Delta\lambda_{\text{max}}$) as expected for an increase in the

Figure 5.1. A) Atomic force microscopy (AFM) image of Au nanoplates on a Si/SiO_x sample after purification by tape. The inset shows the zoom-in image of a nanoplate. AFM images of Au nanoplates purified by taping and then functionalized with anti-IgG (0.26 $\mu\text{g/mL}$) using B) pure MUA, C) 5 mM place-exchanged MUA, and D) 6 mM place-exchanged MUA.

Figure 5.2. LSPR spectra of Au nanoplates purified by sonication and then functionalized with anti-IgG (0.26 $\mu\text{g/mL}$) using A) pure MUA, B) 5 mM place-exchanged MUA, C) 6 mM place-exchanged MUA, and D) pure ME.

refractive index of the environment upon protein binding. For the place-exchanged samples, the shift in the LSPR band was 44 nm and 54 nm for the 5 mM and 6 mM MUA exchange, respectively. The sample of pure ME showed a $\Delta\lambda_{\max}$ of 2 nm. The extinction changes are random and irreproducible from sample to sample, which was not understood at this point of time.

We performed similar experiments on samples synthesized by GS1 and purified by tape and observed a similar response as for those purified for sonication. (See Figures 5.3-5.6 for all spectra on GS1 samples purified by tape and Figures 5.7-5.9 for all data on GS1 sonication samples.)

Figure 5.2 shows the result of 1 sample for each anti-IgG attachment strategy on samples purified by ultrasonication. Figure 5.10 shows a bar graph displaying the average and standard deviation of the $\Delta\lambda_{\max}$ for all methods of synthesis and purification and the three attachment strategies and pure ME control based on at least three samples for each method. The average $\Delta\lambda_{\max}$ was 53 ± 4 nm, 49 ± 13 nm, and 21 ± 5 nm for samples functionalized by 6 mM place-exchange, 5 mM place-exchange, and pure MUA strategies, respectively on GS1 sonicated samples whereas the samples purified by tape showed values of 32 ± 3 nm, 23 ± 1 nm, and 12 ± 1 nm, respectively. Non purified samples synthesized by GS1 showed $\Delta\lambda_{\max}$ values of 9 ± 1 nm, 6 ± 3 nm, and 1 ± 1 nm for the three strategies, respectively. The average $\Delta\lambda_{\max}$ was < 3 nm for the control Au nanoplates functionalized with pure ME. The general increase in $\lambda_{\max}$ for all of samples upon anti-IgG binding is due to a local increase in the refractive index around the Au nanostructure environment as described previously.^{94, 95} The small $\Delta\lambda_{\max}$ change in the

Pure MUA Samples

Figure 5.3. LSPR spectra of three different taped samples of Au nanoplates functionalized with anti-IgG using the pure MUA method and 0.26 $\mu\text{g/mL}$ anti-IgG.

Place-exchange Samples (5 mM MUA)

Figure 5.4. LSPR spectra of three different taped samples of Au nanoplates functionalized with anti-IgG using the place-exchange method (5 mM MUA) and 0.26 $\mu\text{g/mL}$ anti-IgG.

Place-exchange Samples (6 mM MUA)

Figure 5.5. LSPR spectra of three different taped samples of Au nanoplates functionalized with anti-IgG using the place-exchange method (6 mM MUA) and 0.26 $\mu\text{g/mL}$ anti-IgG.

Pure ME samples

Figure 5.6. LSPR spectra of three different taped samples of Au nanoplates functionalized with pure ME and exposed to 0.26 $\mu\text{g/mL}$ anti-IgG.

GS 1 SONICATED SAMPLES

Pure MUA Samples

Figure 5.7. LSPR spectra of six different GS 1 sonicated samples of Au nanoplates functionalized with anti-IgG using the pure MUA method and 0.26 $\mu\text{g/mL}$ anti-IgG.

Place-exchange Samples (5 mM MUA)

Figure 5.8. LSPR spectra of five different “GS 1 sonicated” samples of Au nanoplates functionalized with anti-IgG using the place-exchange method (5 mM MUA) and 0.26 µg/mL anti-IgG.

Place-exchange Samples (6 mM MUA)

Figure 5.9. LSPR spectra of four different “GS 1 sonicated” samples of Au nanoplates functionalized with anti-IgG using the place-exchange method (6 mM MUA) and 0.26 µg/mL anti-IgG.

Figure 5.10. Bar graph showing the average and standard deviation for change in wavelength for Au nanoplate samples purified by GS 1 sonication, GS 1 taped, GS 1 as prepared and then functionalized with anti-IgG using the different strategies and a 0.26 $\mu\text{g/mL}$ anti-IgG concentration.

control experiment importantly confirms that 1) anti-IgG binds specifically to MUA, likely through covalent amide bonds, and 2) we were successful at measuring the optical properties of the glass slide in the same location before and after exposure to anti-IgG. We also note that the maximum $\Delta\lambda_{\text{max}}$ was < 3 nm, for samples of untreated Au nanoplates in air over a one-week period. This reflects the stability of the nanostructures and the precision of the instrument and our sample placement procedure. It also shows that the spectral changes observed for the MUA-containing samples upon anti-IgG binding are significant, while the changes observed for the ME-functionalized Au nanoplates are below the instrument and sample placement precision and therefore insignificant, which indicates minimal non-specific adsorption.

There is clearly a trend in the $\Delta\lambda_{\text{max}}$ with the different attachment strategies following the order of 6 mM place-exchange $>$ 5 mM place-exchange $>$ pure MUA. We believe the different $\Delta\lambda_{\text{max}}$ values for place-exchange and pure MUA samples are due to different anti-IgG binding locations on the Au nanoplates. The AFM images showed anti-IgG bound primarily to edge sites for the place-exchange samples and terrace sites for the pure MUA samples (Figure 5.1). Taken together, the data indicate that the LSPR band is more sensitive to anti-IgG bound to the edge or vertex sites compared to flat terrace sites as predicted previously¹⁰⁷ and as shown by our group on spherical particles.⁴⁷ This was true even though the anti-IgG coverage was larger on the terraces of the Au nanoplates functionalized by the pure MUA strategy compared to the coverage on the edge sites using the 5 mM MUA place-exchange. The larger $\Delta\lambda_{\text{max}}$ values of the 6 mM place-exchange samples relative to the 5 mM samples were due to the larger coverage of anti-IgG on the edge sites for the former (See Figure 5.11 for coverage information). There

Figure 5.11. Plot of average root-mean-square (RMS) roughness of the interior of Au nanoplates (not including edges) as a function of anti-IgG attachment strategy for a concentration of 0.26 µg/mL. The error bars represent the standard deviation measured from three nanoplates. The RMS values are indicative of the coverage of anti-IgG on the flat terrace of the Au nanoplates.

was no clear trend in the change in extinction for the different strategies (see Figure 5.12 for the plot of anti-IgG concentration vs extinction change). The large error bars prevent a conclusive analysis of these results. The irreproducibility could be due to an unknown and uncontrolled phenomenon that drastically affects the absorbance intensity of Au nanoplates or it may be due to an irreproducible loss of Au nanoplates from the surface during soaking in anti-IgG. It is difficult to imagine that a large amount of Au nanoplates detach from the surface during soaking in anti-IgG when the Au nanoplates are stable against taping and sonication though. We believe that further experimentation is necessary to better understand the extinction changes that occur during biomolecular binding to Au nanoplates and other nanostructures. We note that most LSPR spectroscopy sensing studies monitor $\Delta\lambda_{\max}$, rather than extinction, as the signal.^{92, 112}

There is also a trend in the anti-IgG attachment strategies as a function of method of purification. The $\Delta\lambda_{\max}$ followed the order GS1 sonication > GS1 taped > GS1 as prepared when comparing all anti-IgG attachment strategies (Figure 5.10). The order of the shift in $\Delta\lambda_{\max}$ follows the order of $\Delta\lambda_{\max, \text{initial}}$. The initial average $\lambda_{\max}$ was 704 ± 8 nm for GS1 sonicated, 675 ± 54 nm for GS1 taped and 545 ± 4 nm for GS1 as prepared, respectively when comparing the 6 mM MUA place-exchange. It is well known that the bulk RI sensitivity (S_o) increases as the $\Delta\lambda_{\max, \text{initial}}$ increases. This explains the trend in sensitivity for the different purification methods. The large $\Delta\lambda_{\max}$ in the sonicated samples is promising for increasing the sensitivity to protein binding.

Table 5.1 shows the average $\Delta\lambda_{\max}$ for the two different purification strategies along with as prepared samples using GS1 and anti-IgG attachment strategies. The table also shows the purity of the Au nanoplates which was reported previously and the average

Figure 5.12. Bar graphs showing the average and standard deviation for change in normalized extinction on A) samples purified by tape and B) samples purified by sonication before functionalizing with anti-IgG using the different strategies and a 0.26 $\mu\text{g/mL}$ anti-IgG concentration.

Table 5.1. Data table showing the response to anti-IgG for Au nanoplate samples purified by different methods and functionalized by the different strategies.

Purification Strategy	% Au Nanoplates	$\lambda_{\max}$, init(nm)	$\Delta\lambda_{\max}$ Pure MUA	$\lambda_{\max}$, init(nm)	$\Delta\lambda_{\max}$ 5 mM Exch	$\lambda_{\max}$, init(nm)	$\Delta\lambda_{\max}$ 6 mM Exch
GS 1 Taped	90 ± 11	674 ± 21	12 ± 1	644 ± 52	23 ± 1	675 ± 54	32 ± 3
GS 1 Sonication	91 ± 9	743 ± 35	21 ± 5	766 ± 70	49 ± 13	704 ± 8	53 ± 4
GS1 As prepared	23 ± 7	539 ± 14	1 ± 1	547 ± 12	6 ± 3	545 ± 4	9 ± 1

initial $\lambda_{\max}$ of the lowest wavelength peak.¹⁷² In the case of taped and sonicated samples synthesized by GS1, the $\Delta\lambda_{\max}$ followed the order 6 mM exchange > 5 mM exchange > pure MUA as discussed previously. Comparing purification strategies, the average $\Delta\lambda_{\max}$ followed the order GS1 sonicated > GS1 taped > GS1 as prepared samples for all attachment strategies. For example with the 5 mM place-exchange functionalization, $\Delta\lambda_{\max}$ followed the order of GS1 sonication (49 nm) > GS1 taped (23 nm) > GS1 as prepared (6 nm). This order is consistent with previous reports showing that the sensitivity of metal nanostructures to bulk refractive index changes increases as the initial $\lambda_{\max}$ increases to higher wavelengths.^{109, 134, 148, 149} This leads to a larger S_0 value in equations 5.1 as the initial $\lambda_{\max}$ increases. We note that two samples purified by taping, functionalized with pure MUA, and then coated with a very large coverage of anti-IgG from a 250 $\mu\text{g/mL}$ solution exhibited an average $\Delta\lambda_{\max}$ of 40 nm from two samples (Figure 5.13). If we assume 40 nm is the maximum possible shift for the taped samples, then partial attachment of anti-IgG to the edge sites (by 5 or 6 mM MUA place-exchange), led to a signal that is 50-80% of the maximum possible signal (22-32 nm), even though there was much less anti-IgG attached to the surface. This clearly shows that most of the LSPR sensitivity is confined to the edge sites.

Effect of Binding Distance on LSPR Response to Anti-IgG Binding.

Once we determined that the edge sites of the Au nanoplates were more sensitive to local binding and that nanoplates purified by sonication were more sensitive compared to taped or as prepared samples, we decided to determine the effect of binding distance in order to optimize this variable. As shown in equation 5.1, the $\Delta\lambda_{\max}$ (or signal) should

Figure 5.13. LSPR spectra of two different samples of Au nanoplates purified by tapping and then functionalized with anti-IgG using the pure MUA method and 250 $\mu\text{g/mL}$ anti-IgG. This represents the approximate largest shift possible for the Au nanoplates based on complete coating of the nanoplates with anti-IgG at this high concentration.

decrease exponentially with an increase in binding distance due to an exponential decrease in the localized electromagnetic field enhancement. We studied the effect of chain length on the LSPR response of Au nanoplates on a 6 mM place-exchanged sample synthesized by GS1 and purified by sonication. We used three different linkers to control the anti-IgG binding distance, which were 16-mercaptohexadecanoic acid (MHDA), 11-mercaptoundecanoic acid (MUA) and mercaptopropionic acid (MPA). Figure 5.14 shows a schematic representation of anti-IgG attachment using MPA (Frame A), MUA (Frame B) and MHDA (Frame C).

Figure 5.15A shows the LSPR response to anti-IgG bound to MHDA, which showed a $\Delta\lambda_{\max}$ of 43 nm. Under the same exchange conditions, binding to MUA showed a $\Delta\lambda_{\max}$ of 54 nm (Figure 5.15B) and MPA showed a $\Delta\lambda_{\max}$ of 65 nm (Figure 5.15C). According to equation 1 above, $\Delta\lambda_{\max}$ has an exponential dependence with distance from the nanoparticle surface. For Au nanoplates exchanged with MPA, the bound anti-IgG should be closest to the nanoparticle surface and exhibit a larger $\Delta\lambda_{\max}$ compared to anti-IgG bound to the longer MUA and MHDA linkers where the protein should be farther away from the surface. While Figure 5.15 shows only one set of examples Figure 5.16 shows a plot of $\Delta\lambda_{\max}$ vs carbon chain length using the 6 mM place exchange conditions on GS1 sonicated. The $\Delta\lambda_{\max}$ decreases exponentially with increasing chain length as expected. The error bars represents the standard deviations from at least three samples (See Figures 5.17-5.20 for all spectra using 5 and 6 mM MHDA and MPA on samples purified by sonication using GS1). We also performed similar experiments on samples of GS1 as prepared and those prepared by GS1 and purified by tape and observed similar results. (See Figures 5.21-5.24 for all spectra using 5 and 6 mM MHDA and MPA on

samples purified by tape using GS1 and Figures 5.25-5.28 of for all spectra using 5 and 6 mM MHDA and MPA on GS1 as prepared samples).

Figure 5.14. Schematic representation of anti-IgG attachment to edges of Au nanoplates using three different chain lengths MPA (A), MUA (B) and MHDA (C).

Figure 5.15. LSPR spectra of Au nanoplates purified by sonication before and after attachment of anti-IgG using the A) 6 mM MHDA B) 6 mM MUA and C) 6 mM MPA place-exchange method and 0.26 $\mu\text{g/mL}$ anti-IgG.

Figure 5.16. Plot showing the average and standard deviation for change in wavelength for Au nanoplate samples purified by sonication using GS 1 and then functionalized with anti-IgG using the 6 mM place-exchange strategy with the three different carbon chain lengths (MHDA, MUA, MPA) and a 0.26 $\mu\text{g/mL}$ anti-IgG concentration.

Place-exchange Samples (5 mM MHDA)

Figure 5.17. LSPR spectra of three different “GS 1 sonicated” samples of Au nanoplates functionalized with anti-IgG using the place-exchange method (5 mM MHDA) and 0.26 $\mu\text{g/mL}$ anti-IgG.

Place-exchange Samples (6 mM MHDA)

Figure 5.18. LSPR spectra of three different “GS 1 sonicated” samples of Au nanoplates functionalized with anti-IgG using the place-exchange method (6 mM MHDA) and 0.26 $\mu\text{g/mL}$ anti-IgG.

Place-exchange Samples (5 mM MPA)

Figure 5.19. LSPR spectra of three different “GS 1 sonicated” samples of Au nanoplates functionalized with anti-IgG using the place-exchange method (5 mM MPA) and 0.26 µg/mL anti-IgG.

Place-exchange Samples (6 mM MPA)

Figure 5.20. LSPR spectra of three different “GS 1 sonicated” samples of Au nanoplates functionalized with anti-IgG using the place-exchange method (6 mM MPA) and 0.26 µg/mL anti-IgG.

Place-exchange Samples (5 mM MHDA)

Figure 5.21. LSPR spectra of three different taped samples of Au nanoplates functionalized with anti-IgG using the place-exchange method (5 mM MHDA) and 0.26 $\mu\text{g/mL}$ anti-IgG.

Place-exchange Samples (6 mM MHDA)

Figure 5.22. LSPR spectra of three different taped samples of Au nanoplates functionalized with anti-IgG using the place-exchange method (6 mM MHDA) and 0.26 $\mu\text{g/mL}$ anti-IgG.

Place-exchange Samples (5 mM MPA)

Figure 5.23. LSPR spectra of three different taped samples of Au nanoplates functionalized with anti-IgG using the place-exchange method (5 mM MPA) and 0.26 $\mu\text{g/mL}$ anti-IgG.

Place-exchange Samples (6 mM MPA)

Figure 5.24. LSPR spectra of three different taped samples of Au nanoplates functionalized with anti-IgG using the place-exchange method (6 mM MPA) and 0.26 $\mu\text{g/mL}$ anti-IgG.

Place-exchange Samples (5 mM MHDA)

Figure 5.25. LSPR spectra of five different “GS1” samples of Au nanoplates functionalized with anti-IgG using the place-exchange method (5 mM MHDA) and 0.26 $\mu\text{g/mL}$ anti-IgG.

Place-exchange Samples (6 mM MHDA)

Figure 5.26. LSPR spectra of three different “GS 1” samples of Au nanoplates functionalized with anti-IgG using the place-exchange method (6 mM MHDA) and 0.26 $\mu\text{g/mL}$ anti-IgG.

Place-exchange Samples (5 mM MPA)

Figure 5.27. LSPR spectra of five different “GS 1” samples of Au nanoplates functionalized with anti-IgG using the place-exchange method (5 mM MPA) and 0.26 $\mu\text{g/mL}$ anti-IgG.

Place-exchange Samples (6 mM MPA)

Figure 5.28. LSPR spectra of five different “GS 1” samples of Au nanoplates functionalized with anti-IgG using the place-exchange method (6 mM MPA) and 0.26 $\mu\text{g/mL}$ anti-IgG.

Location-Dependent IgG Sensing.

We performed an immunoassay with human IgG after immobilizing anti-IgG on Au nanoplate samples synthesized by GS1 and purified by tapping. Figure 5.29 shows the optical changes in the LSPR spectra for IgG detection after immobilizing anti-IgG on the Au nanoplates using 6 mM MUA place-exchange (frame A) and pure MUA (frame B). The black curve represents the spectrum before anti-IgG immobilization. The LSPR band after immobilizing 0.26 $\mu\text{g/mL}$ anti-IgG (red curve) exhibited a shift of 28 nm while the LSPR band shifted only by 14 nm on a pure MUA treated sample as discussed previously. Exposure to a 10 pg/mL solution of IgG on the exchanged sample lead to a further shift in the LSPR band to longer wavelengths by ~ 9 nm (green curve). This is among the lowest concentration detected by LSPR for proteins to date.¹²⁸ The immunoassay performed with the anti-IgG bound to pure MUA treated Au nanoplates responded only to 1 ng/mL (1000 pg/mL) IgG with a shift of 7 nm. This is a 2 order of magnitude improvement by controlling the binding location.

Selective edge attachment of 12 nm Au seeds: Because protein molecules are very small it was difficult sometimes to visualize anti-IgG on the nanoplates. We immobilized 12 nm seed on Au nanoplates by place-exchanging the ME functionalized nanoplate sample with 6 mM octanedithiol (ODT) before placing the sample in a solution of Au seed particles. Figure 5.30A shows a schematic procedure for immobilizing seed nanoparticles on the nanoplates. Figures 5.30 B through D shows AFM images of the edge attached 12 nm seed nanoparticles. This also confirms that place-exchange selectively occurs at the edge sites.

Figure 5.29. LSPR spectra showing A) 0.01 ng/mL IgG detection on a 6 mM MUA exchanged sample and B) 1 ng/mL IgG on a pure MUA treated sample after immobilizing 0.26 $\mu\text{g/mL}$ anti-IgG.

Figure 5.30. A) Scheme showing selective edge attachment of Au seeds on the nanoplates. B), C), and D) are the AFM images of 12 nm seed particles on Au nanoplates.

5.4. CONCLUSIONS

There are two major advancements reported in this work. This represents a major improvement over our previous work, which showed the optical response of highly disperse samples of nanoplates and spherical particles. The $\Delta\lambda_{\max}$ values from this work along with the AFM images confirm that the edge sites are more sensitive to anti-IgG binding compared to terrace sites and also shows that pure Au nanoplates are more sensitive compared to spherical Au NPs. Spherical Au NPs previously displayed $\Delta\lambda_{\max}$ values of 5-6 nm and 9-10 nm for place-exchange samples using 5 mM and 6 mM MUA, respectively. Under identical conditions, the purified Au nanoplates displayed average $\Delta\lambda_{\max}$ values of 23 nm and 32 nm, respectively, for taped GS1 samples and 49 nm and 53 nm for sonicated GS1 samples. The second discovery was that the exchange performed with short carbon chain length (MPA) showed average $\Delta\lambda_{\max}$ values of 61 nm where as MUA showed an average $\Delta\lambda_{\max}$ of 53 nm and MHDA showed an average $\Delta\lambda_{\max}$ value of 42 nm for a 6 mM exchange sample conditions. We also detected a 0.01 ng/mL IgG solution optically using Au nanoplates functionalized with anti-IgG through the optimized place-exchange procedure. The AFM images reveal that only a few anti-IgG molecules bound to edge sites lead to a large $\Delta\lambda_{\max}$, showing that single biomolecule detection is a real possibility. The experimental demonstration of controlled binding of biomolecules at the edge sites of Au nanostructures and correlation between LSPR response and binding location allows us to obtain a better fundamental understanding of LSPR based detection in order to optimize these materials for biosensing applications. Controlled binding to edge sites should also impact applications in SERS-based sensing.

There are still several questions to answer regarding the effect of local protein binding on the extinction of Au nanoplates, as the results were sporadic.

CHAPTER VI

SUB-PICOMOLAR LABEL FREE PROTEIN DETECTION WITH SURFACE ATTACHED GOLD NANOPATES BY LOCALIZED SURFACE PLASMON RESONANCE SPECTROSCOPY

Here we report the label-free detection of a model protein, human IgG, by monitoring the Localized Surface Plasmon Resonance (LSPR) band of surface attached Au nanoplates. Human IgG detection occurred in an immunoassay format with human anti-IgG bound to the Au nanoplates capturing IgG in solution and producing a shift in the LSPR band ($\lambda_{\max}$) at concentrations down to 1 pg/mL (< 0.1 pM). The dynamic range is 0.001 to 100 ng/mL where the signal change ($\Delta\lambda_{\max}$) ranged from 12 to 70 nm. No LSPR response occurred for control experiments with buffer only or nonspecific antigen goat IgG at low and medium concentrations of goat-IgG, although small signals arose at concentrations of 1000 ng/mL. The keys to high sensitivity include purification of Au nanoplates ($\sim 90\%$) with an initial $\lambda_{\max}$ of ~ 700 nm, the selective edge-functionalization of the nanoplates with human anti-IgG, and monitoring the LSPR band in air before and after IgG exposure. A drawback of the detection strategy is the long response time (~ 24 hrs), which stirring did not improve.

6.1. INTRODUCTION

It is well-known that sharp edges and corners of nanostructures exhibit much larger electric field enhancement compared to smooth terrace regions.⁵⁸ For example, the electric field enhancement is large at the ends of nanorods, vertices of triangular nanoplates, points of branched structures, or within coupled nanoparticle pairs.^{58, 156} These regions are considered to be “hot spots” that are highly sensitive to molecules binding in the vicinity much in the same way that these “hot spots” are responsible for surface enhanced Raman scattering (SERS) of Raman active molecules in their vicinity.

We previously showed the controlled binding of anti-IgG to the edge and vertex sites of spherical Au nanoparticles and purified Au nanoplates synthesized directly on glass and Si/SiO_x surfaces. The LSPR response of edge functionalized antibodies on Au nanoplates was dramatically larger than terrace-bound antibodies. In our previous work we reported that Au nanoplate samples purified by sonication showed a greater LSPR response compared to non-purified samples with mostly Au spheres and tape-purified samples since the initial λ_{max} is larger. We also previously optimized the LSPR response to protein binding by using a short chain 3-mercaptopropionic acid (MPA) to immobilize antiIgG on the surface. Here we report the detailed LSPR response of optimized Au nanoplate samples to various concentrations of specific human IgG compared to nonspecific IgG. We provide the limit of detection, specificity, and effect of stirring on the response time, which is generally very slow at low concentrations.

6.2. EXPERIMENTAL METHODS

Chemicals. Citric acid trisodium salt was purchased from Bio-rad laboratories. L-ascorbic acid (99%), sodium borohydride (98.5%), cetyltrimethylammonium bromide (CTAB), 3-mercaptopropionic acid (MPA), 2-mercaptoethanol (ME), N-hydroxy succinimide (NHS), and silver nitrate (AgNO_3) were purchased from Sigma-Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Pierce and 95 % mercaptopropyltrimethoxy silane (MPTMS) was purchased from Alfa Aesar. 2-propanol was purchased from Pharmco and toluene from Honeywell. Stock solutions of 2.5 mg/mL antibody anti-human-IgG and 10 mg/mL antigen human-IgG, 5 mg/mL goat-IgG, and PBS were purchased from Sigma-Aldrich. HAuCl_4 was synthesized from solid Au.

Synthesis of Au Nanostructures Directly on Surfaces. Au nanostructures were synthesized directly on glass and silicon surfaces, purified and functionalized with anti-IgG for IgG sensing in a similar way as discussed in chapter II of experimental section.

6.3. RESULTS AND DISCUSSION

LSPR Sensing of IgG. Figure 6.1 shows an SEM image of the nanoplate sample purified by sonication. The dark region represents the Si/SiO_x and the grey features represents the nanoplates. Glass slides coated with Au nanoplates were carefully marked to measure the extinction from the same location before and after human anti-IgG functionalization and subsequent exposure to human IgG to monitor the LSPR response to local protein binding. All of the samples displayed an initial λ_{max} from 680 to 750 nm

Figure 6.1. SEM image of an Au nanoplate sample on the surface of silicon after purification by sonication.

before anti-IgG functionalization. As mentioned in the previous chapters, the sensitivity of the LSPR $\lambda_{\max}$ to the environment increases with increasing initial $\lambda_{\max}$, so we studied samples with initial $\lambda_{\max}$ values over this narrow range. We edge functionalized the Au nanoplates with anti-human IgG by assembly of ME on the surface, place-exchange of ME with 6 mM MPA, and finally EDC coupling of human anti-IgG as described previously in chapter V. We previously found that these conditions were optimal for IgG sensing with Au nanoplates.

Figure 6.2 shows the general sensing scheme for IgG sensing after immobilizing anti-IgG on purified Au nanoplates.

Figure 6.3A shows the LSPR spectra of Au nanoplates on glass before (black plot) and after (red plot) anti-IgG functionalization and after exposure to 0.001 ng/mL human IgG for 24 hrs (green plot). The shift in the LSPR band after anti-IgG functionalization ($\Delta\lambda_{\max1}$) was 46 nm and after exposure to IgG ($\Delta\lambda_{\max2}$) was 13 nm. The sample showed a shift in $\lambda_{\max}$ after anti-IgG and IgG binding due to a change in the refractive index around the Au nanoplates, consistent with our previous results. Figure 6.3 Frames B through F, show similar LSPR spectra for Au nanoplate samples after anti-IgG functionalization and exposure to human IgG at concentrations ranging from 0.1 to 100 ng/mL. In all cases, $\Delta\lambda_{\max1}$ was similar, ranging from 46 to 70 nm. The $\Delta\lambda_{\max2}$ ranged from 13 to 71 nm, increasing with increasing human IgG concentration.

Figure 6.4 shows a plot of $\Delta\lambda_{\max}$ vs log (IgG) for Au nanoplates after exposure to human and goat IgG. The error bars represent the standard deviation from at least three samples, except at 0.001 ng/mL, for which we observed a response only twice out of 5

Figure 6.2. Scheme showing the synthesis and purification of Au nanoplates directly on surfaces for LSPR sensing of antigen after antibody immobilization.

Figure 6.3. LSPR spectra of Au nanoplates before (black) and after edge functionalization with human anti-IgG and after subsequent exposure to human IgG (green) at concentrations of A) 0.001 ng/mL, B) 0.01 ng/mL, C) 0.1 ng/mL, D) 1.0 ng/mL, E) 10 ng/mL, and F) 100 ng/mL.

Figure 6.4. Plot of wavelength shift vs log (IgG) for specific human IgG and nonspecific goat IgG.

samples. The average $\Delta\lambda_{\text{max}}$ after IgG exposure was 12.5 ± 0.7 nm, 23.7 ± 5.5 nm, 35 ± 5 nm, 50.7 ± 3.2 nm, 65.3 ± 4.9 nm, 69.5 ± 2.1 nm and 70 ± 1.4 nm for 0.001, 0.01, 0.1, 1, 10, 100 and 1000 ng/mL respectively. The $\Delta\lambda_{\text{max}}$ values after IgG exposure increased with increasing IgG concentration from 0.001 ng/mL to 100 ng/mL and then saturated beyond 100 ng/mL IgG. Further increase in IgG concentration to 1000 ng/mL showed a similar response, indicating that anti-IgG binding sites become saturated. Figure 6.4 also shows the response for nonspecific antigen goat-IgG at various concentrations from 0.01 ng/mL to 1000 ng/mL. The $\Delta\lambda_{\text{max}}$ for concentrations from 0.01 ng/mL to 100 ng/mL was less than or equal to 3 nm (insignificant) while that at 1000 ng/mL goat IgG was 11 ± 1.4 nm, which is due to some nonspecific binding at high concentrations. The plot of log of IgG concentration vs wavelength shift calibration plot is not linear. This could be due to the varied number of binding sites of IgG. This need to be understood further.

Figure 6.5 shows the LSPR response for control samples of pure ME-functionalized Au nanoplates exposed to anti-IgG (Frame A), and anti-IgG edge functionalized Au nanoplates exposed to buffer only (Frame B) and nonspecific goat IgG (Frame C). The $\Delta\lambda_{\text{max}}$ with the pure ME-coated nanoplates when exposed to anti-IgG for 12-15 hrs was 2 nm. This confirms that anti-IgG binds specifically to MPA, likely through covalent amide bonds, and that we were successful at measuring the optical properties of the glass slide in the same location, since the spectrum is nearly identical before and after anti-IgG exposure. We also note that the maximum $\Delta\lambda_{\text{max}}$ was < 3 nm for samples of untreated Au nanoplates in air. This reflects the stability of the nanostructures and the precision of the

Figure 6.5. LSPR spectra of A) ME-coated Au nanoplates before and after exposure to pH 7.4 phosphate buffered 0.26 $\mu\text{g/mL}$ human anti-IgG for 24 hours and human anti-IgG edge functionalized Au nanoplates exposed to B) pH 7.4 phosphate buffer and C) pH 7.4 phosphate buffered 0.01 ng/mL goat IgG for 24 hours.

instrument and our sample placement. Any signal below 3 nm would be considered insignificant. The control experiments in Frames B and C displayed an insignificant $\Delta\lambda_{\max}$ of 2 nm and 3 nm, which shows that the nanoplates are stable in buffer only overnight, the anti-human IgG does not bind goat IgG, and that we were successful in measuring the spectra at the same location of the glass slide. The human anti-IgG edge functionalized Au nanoplates are clearly stable, highly selective, and highly sensitive to the target protein. These samples could easily be employed for the detection of more relevant proteins, such as cancer markers.

Effect of stirring on the LSPR response of IgG binding to anti-IgG

We also studied the effect of stirring on IgG binding to anti-IgG functionalized Au nanoplates in order to decrease the response time. Figure 6.6 shows the LSPR spectrum of an Au nanoplate sample before (black), after (red) anti-IgG functionalization and after stirring for 12 hours in a 0.01 ng/mL pH 7.4 phosphate buffered IgG solution (green plot). The sample showed a $\Delta\lambda_{\max}$ of 60 nm after anti-IgG edge functionalization consistent with other samples, and a $\Delta\lambda_{\max}$ of 12 nm after stirring for 12 hrs in IgG. A nonstirred sample after 24 hrs showed a $\Delta\lambda_{\max}$ value of 23.7 ± 5.5 nm (Figure 6.3). No change in the LSPR response occurred when the solution was stirred for less than 12 hrs. From this experiment we conclude that stirring does not significantly reduce the response time at this concentration. Some other strategy will be needed to shorten the long analysis times.

Figure 6.6. LSPR spectra showing the effect of stirring on IgG binding to anti-IgG

6.4. CONCLUSIONS

In summary, we studied the collective LSPR response of pure anti-IgG functionalized Au nanoplates to IgG at concentrations from 0.001 to 1000 ng/mL. The $\Delta\lambda_{\max}$ ranged from 12.5 ± 0.7 nm to 69.5 ± 2.1 nm over these concentrations, increasing with increasing IgG concentration until saturation above approximately 100 ng/mL IgG. Control experiments with buffer only and nonspecific goat IgG showed no LSPR response which confirms the selectivity to human-IgG. Stirring appeared to have a minimal effect on the long 24 hr analysis time. The 1 pg/mL (< 0.1 pM) concentration detected here is the lowest LSPR detection limit reported for proteins to our knowledge. The high sensitivity to protein binding is due to 1) purified Au nanoplates with initial $\lambda_{\max}$ in air of 650 – 750 nm, 2) selective edge-functionalization of the nanoplates with anti-IgG, and 3) monitoring the LSPR in air before and after binding. This label-free, highly sensitive and specific protein detection strategy could find use in a number of biomedical applications, such as the detection of cancer markers. Future research will explore applications and reduce the analysis time.

CHAPTER VII

LOCALIZED SURFACE PLASMON RESONANCE SENSING WITH Ag-COATED Au NANOPATES GROWN DIRECTLY ON SURFACES

Here we report the synthesis of Ag-coated Au nanoplates (Au/Ag) directly on the surface of glass and Si/SiO^x and the localized surface plasmon resonance (LSPR) response to global refractive index changes and local biomolecule binding. The Au/Ag nanoplate samples displayed a global refractive index sensitivity of 439 - 651 nm/RIU with an initial wavelength of 638 nm. The initial single LSPR band split into two peaks in the presence of various liquids. The local LSPR response of Ag/Au nanoplates functionalized with anti-IgG to human IgG binding increased with increasing IgG concentration from 1 pg/mL to 100 ng/mL where the signal saturates. The average change in λ_{max} ($\Delta\lambda_{\text{max}}$) is 17.5 ± 0.7 nm for binding at 1 pg/mL IgG and 75.5 ± 2.1 nm for binding at 100 ng/mL IgG concentration. Control experiments with buffer only and nonspecific antigen goat IgG (< 100 ng/mL) showed no LSPR response; there is a small shift in the LSPR band after exposure to 1000 ng/mL goat IgG. The key to high sensitivity is the purity of the nanoplates and controlled edge-functionalization of the plates with anti-IgG. The Au/Ag nanoplates are slightly more sensitive compared to Au nanoplates to global RI changes, but not to local protein binding.

7.1. INTRODUCTION

We previously showed the controlled binding of anti-IgG to the edge and vertex sites of spherical Au nanoparticles and purified Au nanoplates synthesized directly on glass and Si/SiO_x surfaces. Here we studied the LSPR response of IgG binding to anti-IgG on silver coated Au (Au/Ag) nanoplates. These nanostructures showed greater bulk RI sensitivity in solvents of varying RI than the Au nanoplates samples. The local RI sensitivity as a result of IgG response was similar to the Au nanoplate samples.

7.2. EXPERIMENTAL

Chemicals. Citric acid trisodium salt was purchased from Bio-rad laboratories. L-ascorbic acid (99%), sodium borohydride (98.5%), cetyltrimethylammonium bromide (CTAB), 3-mercaptopropionic acid (MPA), 2-mercaptoethanol (ME), N-hydroxy succinimide (NHS), and silver nitrate (AgNO₃) were purchased from Sigma-Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Pierce and 95 % mercaptopropyltrimethoxy silane (MPTMS) was purchased from Alfa Aesar. 2-propanol was purchased from Pharmco and toluene from Honeywell. Stock solutions of 2.5 mg/mL antibody anti-human-IgG and 10 mg/mL antigen human-IgG, 5 mg/mL goat-IgG, and PBS were purchased from Sigma-Aldrich. HAuCl₄ was synthesized from solid Au.

Synthesis of Au/Ag Nanoplates. Glass and Si/SiO_x slides were first cut and cleaned in piranha solution (1:3 H₂O₂:H₂SO₄) for 10-15 min. (**Caution: this solution, piranha, is a strong oxidizing agent that reacts violently with organics.**) After rinsing with water

and drying under N₂, the substrates were functionalized with MPTMS by heating them just below boiling in a solution containing 10 mL of 2-propanol, 100 μ L of MPTMS, and a few drops of water for about 30 min. After rinsing with 2-propanol and drying under N₂, the MPTMS-functionalized glass and Si/SiO_x slides were placed in an aqueous solution of 3-5 nm diameter citrate-stabilized Au nanoparticles (“seeds”) for 15 minutes, which leads to their attachment to the thiol functionality of MPTMS through a strong Au-thiolate interaction. After rinsing with water, the substrates containing immobilized Au seed nanoparticles were then placed in a freshly prepared solution containing 9 mL of 0.1 M CTAB, 450 μ L of 0.01 M HAuCl₄, and 50 μ L of 0.01 M ascorbic acid for 1 h. The samples were rinsed with water and dried under nitrogen before further use. The as-synthesized nanostructures had ~ 23 % Au nanoplates and 67 % spherical particles. The nanoplate samples were purified by ultrasonication in a glass vial containing 10 mL of nanopure water for 2-5 minutes. This led to samples with > 90 % Au nanoplates. The substrate was removed from the vial, washed thoroughly with nanopure water, and dried under nitrogen. The resulting Au nanoplate sample was then placed in a freshly prepared Ag growth solution which results in reduction of Ag⁺ in solution onto the Au nanoplates on the surface. The Ag growth solution consists of 10 mL of 0.08 M CTAB in pH 10.6 phosphate buffer, 250 μ L of 0.01 M AgNO₃ and 500 μ L of 0.1 M ascorbic acid.¹⁵⁷ The Au/Ag nanoplates are functionalized with anti-IgG for IgG sensing in a similar way as discussed in chapter II of experimental section.

7.3. RESULTS AND DISCUSSION

Figure 7.1A and 7.1B show SEM images of the sample of purified Au nanoplates before and after Ag deposition and the inset shows a zoom-in of the plates. The dark region represents the Si/SiO_x surface and the grey features represent the Au or Au/Ag nanoplates. The nanoplates after Ag deposition in Figure 7.1B appear very similar to those before Ag deposition shown in Figure 7.1A showing that the Ag deposition proceeded uniformly on the surface of the Au nanoplates. The average width of the nanoplates determined by SEM before Ag deposition was 182 ± 49 nm based on measurements of 55 nanoplates from 3 samples and 211 ± 56 nm after Ag deposition based on 34 nanoplates from two different samples. This uniform deposition and ~ 30 nm increase in width is consistent with that observed by Slawinski *et al.* for Ag growth on Au nanorods.¹⁵⁷ The increased average width suggests that Ag deposition occurred on the surface of the Au nanoplates, but is not conclusive due to the large size deviation. Figure 7.1C shows the LSPR spectrum before (red) and after (purple) Ag deposition on Au nanoplates. The peak at 760 nm represents the wavelength of maximum extinction (λ_{max}) for Au nanoplates purified by sonication as reported by our group previously. The λ_{max} shifted to 622 nm after Ag deposition. The 138 nm blue shift and increase in extinction after Ag growth is expected based on the literature. The LSPR spectrum together with the SEM images confirm that Ag deposition occurred on the Au nanoplates.

Figure 7.1. SEM image of Au nanoplates on a Si/SiO_x surface A) before Ag deposition and B) after Ag deposition. C) LSPR spectra of the Au nanoplates before and after Ag deposition.

Global Refractive Index Sensitivity of Au/Ag nanoplates.

We measured the global refractive index sensitivity of Au/Ag nanoplates synthesized directly on glass by placing the sample in liquids of varying refractive index and measuring the LSPR spectrum. Figure 7.2A shows the LSPR spectra of Au/Ag nanoplates synthesized on glass and placed in air, water, 2-propanol, toluene and then back in air. As the refractive index of the solvent increased the LSPR λ_{max} red shifted to longer wavelengths. The LSPR band also interestingly split into two peaks when placed in higher RI solvents. This could be due to different populations of nanoplates on the glass surface responding differently to their environment. The spectrum in air before exposure to the solvents matches the spectrum after exposure to the different solvents, indicating that the nanoplate samples were stable and the spectra were from the same region of the glass sample. Figure 7.2B shows a plot of the LSPR λ_{max} as a function of the RI of the solvent. The slope of the plot gives the RI sensitivity of the Au/Ag nanoplate sample. From the slope, the RI sensitivity was 651 nm/RIU for the Au/Ag nanoplate sample based on the position of the second peak and 439 nm/RIU based on the first peak with an initial λ_{max} of 638 nm in air. The RI sensitivity of Au nanoplates with an initial λ_{max} of 677 nm in air was only 195 nm/RIU in comparison, which shows that the Au/Ag nanoplates samples are more sensitive than Au nanoplates. The deposition of Ag on to the Au is a simple method for improving the RI sensitivity of the nanoplates.

Sensitivity to Local Protein Binding. Glass slides coated with Au/Ag nanoplates were carefully marked to measure the extinction from the same location before and after human anti-IgG functionalization and subsequent exposure to human IgG to monitor the LSPR response to local protein binding. All of the samples displayed an initial

Figure 7.2. A) LSPR spectra of Au/Ag nanoplates on glass in air and in solvents of different refractive index as indicated. B) A plot of the LSPR $\Delta\lambda_{\max}$ as a function of the solvent refractive index for the sample with a $\lambda_{\max}$ in air of 638 nm.

$\lambda_{\max}$ from 580 to 700 nm before anti-IgG functionalization. As mentioned in the previous chapters, the sensitivity of the LSPR $\lambda_{\max}$ to the environment increases with increasing initial $\lambda_{\max}$, so we studied samples with an initial $\lambda_{\max}$ value over this narrow range. We edge functionalized the Au/Ag nanoplates with anti-human IgG by assembly of ME on the surface, place-exchange of ME with 6 mM MPA, and finally EDC coupling of human anti-IgG as described previously. We previously found that these conditions were optimal for IgG sensing with Au nanoplates.

Figure 7.3 shows the general sensing scheme for IgG sensing after immobilizing anti-IgG on Au/Ag nanoplates.

Figure 7.4A shows the LSPR spectra of Au/Ag nanoplates on glass before (black plot) and after (red plot) anti-IgG functionalization and after exposure to 0.001 ng/mL human IgG for 24 hrs (green plot). The shift in the LSPR band after anti-IgG functionalization ($\Delta\lambda_{\max1}$) was 50 nm and after exposure to IgG ($\Delta\lambda_{\max2}$) was 17 nm. The sample showed a shift in $\lambda_{\max}$ after anti-IgG and IgG binding due to a change in the refractive index around the Au/Ag nanoplates, consistent with our previous results using Au nanoplates. Figure 7.4 Frames B through F, show similar LSPR spectra for Au/Ag nanoplate samples after anti-IgG functionalization and exposure to human IgG at concentrations ranging from 0.1 to 100 ng/mL. In all cases, $\Delta\lambda_{\max1}$ was similar, ranging from 45 to 58 nm. The $\Delta\lambda_{\max2}$ ranged from 17 to 70 nm, increasing with increasing human IgG concentration.

Figure 7.5 shows a plot of $\Delta\lambda_{\max}$ vs log [IgG] for Au/Ag and Au nanoplates after exposure to human and goat IgG. The error bars represent the standard deviation from at least three samples, except at 0.001 ng/mL, for which we observed a response only twice

Figure 7.3. Scheme showing the synthesis of Au/Ag nanoplates directly on surfaces for LSPR sensing of antigen after antibody immobilization.

Figure 7.4. LSPR spectra of Au/Ag nanoplates before (black) and after edge functionalization with human anti-IgG and after subsequent exposure to human IgG (green) at concentrations of A) 0.001 ng/mL, B) 0.01 ng/mL, C) 0.1 ng/mL, D) 1.0 ng/mL, E) 10 ng/mL, and F) 100 ng/mL.

Figure 7.5. Plot of LSPR band wavelength shift for human anti-IgG edge functionalized Au/Ag and Au nanoplates as a function of log (IgG) for exposure to specific human IgG and nonspecific goat IgG

out of 4 samples. The average $\Delta\lambda_{\text{max}}$ after IgG exposure was 17.5 ± 0.7 nm, 28.3 ± 1.5 nm, 37.3 ± 2.3 nm, 49 ± 2.6 nm, 60.3 ± 2.1 nm, 75.5 ± 2.1 nm and 73.5 ± 2.1 nm for 0.001, 0.01, 0.1, 1, 10, 100 and 1000 ng/mL respectively. The $\Delta\lambda_{\text{max}}$ values after IgG exposure increased with increasing IgG concentration from 0.001 ng/mL to 100 ng/mL and then saturated beyond 100 ng/mL IgG. Further increase in IgG concentration to 1000 ng/mL showed a similar response, indicating that anti-IgG binding sites become saturated. Figure 7.5 also shows the response for nonspecific antigen goat-IgG at various concentrations from 0.1 ng/mL to 1000 ng/mL. The $\Delta\lambda_{\text{max}}$ for concentrations from 0.1 ng/mL to 100 ng/mL was less than or equal to 3 nm (insignificant) while that at 1000 ng/mL goat IgG was 11 ± 1.4 nm, which is due to some nonspecific binding at high concentrations. The comparison between Au and Au/Ag nanoplates shows a similar sensitivity to both

Figure 7.6 shows the LSPR response for control samples of pure ME-functionalized Au/Ag nanoplates exposed to anti-IgG (Frame A), and anti-IgG edge functionalized Au/Ag nanoplates exposed to buffer only (Frame B) and nonspecific goat IgG (Frame C). The $\Delta\lambda_{\text{max}}$ with the pure ME-coated nanoplates when exposed to anti-IgG for 12-15 hrs was 0 nm. This confirms that anti-IgG binds specifically to MPA, likely through covalent amide bonds, and that we were successful at measuring the optical properties of the glass slide in the same location, since the spectrum is nearly identical before and after anti-IgG exposure. We also note that the maximum $\Delta\lambda_{\text{max}}$ was < 3 nm for samples of untreated Au/Ag nanoplates in air. This reflects the stability of the nanostructures and the precision of the instrument and our sample placement. Any signal below 3 nm is insignificant. The control experiments in Frames B and C displayed an insignificant

Figure 7.6. LSPR spectra of A) ME-coated Au/Ag nanoplates before and after exposure to pH 7.4 phosphate buffered 0.26 $\mu\text{g/mL}$ human anti-IgG for 24 hours and human anti-IgG edge functionalized Ag/Au nanoplates exposed to B) pH 7.4 phosphate buffer and C) pH 7.4 phosphate buffered 0.01 ng/mL goat IgG for 24 hours.

$\Delta\lambda_{\text{max}}$ of 1 nm, which shows that the nanoplates are stable in buffer only overnight, the anti-human IgG does not bind goat IgG, that again we were successful in measuring the spectra at the same location of the glass slide. The human anti-IgG edge functionalized Au/Ag nanoplates are clearly stable, highly selective, and highly sensitive to the target protein. These samples could be employed for the detection of more relevant proteins, such as cancer markers.

7.4. CONCLUSIONS

In summary, we synthesized Ag-coated Au nanoplates directly on glass and Si/SiO_x by seed-mediated growth directly on surfaces. The synthesized Au/Ag nanoplates showed a global refractive index sensitivity of 439-651 nm/RIU and local response to IgG binding at concentrations ranging from 10 pg/mL to 100 ng/mL when edge functionalized with anti-IgG. The detection of 1 pg/mL demonstrated here is the lowest concentration reported for IgG to our knowledge and clearly at clinically relevant protein concentrations. The high sensitivity is due to 1) the purification of the nanoplates, 2) controlled functionalization of anti-IgG at edge sites, and 3) use of a short linker for anti-IgG (MPA). The deposition of Ag on the nanoplates improves the global RI sensitivity but not to local IgG binding and the anti-IgG receptor is highly selective. Future experiments will be aimed at detecting cancer markers in biologically relevant media and decreasing the response time.

CHAPTER VIII

SUMMARY AND CONCLUSIONS

Selective attachment of antibodies to the edges of nanostructures can be used to detect analyte molecules at very low concentrations with high sensitivity and selectivity. Chapter III shows selective attachment of antibodies to the edges of Au nanostructures that are synthesized directly on surfaces using a seed-mediated method. The samples contained approximately 23 % nanoplates and 77 % spherical nanoparticles. The edge attached antibodies showed enhanced optical properties relative to terrace-bound antibodies. We attached antibodies selectively to the edges by place-exchanging the ME with MUA. Increasing MUA concentrations during exchange increased the number of antibodies at the edges and increased the shift in the LSPR band. The greater sensitivity of the edge sites is due to strong plasmon fields at these sites. While we obtained AFM data from Au nanoplates and optical data from spherical particles, we still lacked direct optical data from Au nanoplates.⁴⁷ The spherical particles could detect IgG concentrations as low as 0.1 ng/mL.

Chapter IV describes the purification of Au nanoplates directly on surfaces that resulted in removing the spherical particles, producing substrates with > 90 % Au nanoplates. The samples were purified by two methods, 1) by using an adhesive tape, and 2) by ultrasonication of the sample. We selectively attached anti-IgG to the edges of nanoplates and detected 0.01 ng/mL IgG concentrations. The $\Delta\lambda_{\text{max}}$ depends on the

method of purification. Sonicated samples showed greater $\Delta\lambda_{\max}$ values to anti-IgG binding than taped samples, which in turn was greater than non-purified samples. We also used a second method to synthesize Au nanoplates directly on surfaces with a 44 % nanoplate yield before purification. In chapter V we studied the effect of the linker chainlength during place exchange and optimized the LSPR response to protein binding by using MPA as a linker. We correlated the optical properties with AFM images as a function of binding location directly on the nanoplates and observed greater LSPR responses to edge attached anti-IgG compared to terrace attached anti-IgG molecules. The edge functionalized anti-IgG could be used to detect IgG concentrations as low as 0.01 ng/mL where as terrace bound anti-IgG could only detect 1 ng/mL IgG with a similar LSPR response.

Detailed IgG LSPR response was described in Chapter VI using edge-exchanges MPA as a linker and nanoplate purified by sonication. No change in LSPR response was observed for nonspecific goat IgG and buffer-only solution whereas specific antigen IgG showed responses to 0.001 ng/mL. We increased the RI sensitivity of the Au nanoplates by depositing Ag on top of Au in Chapter VII, but the sensitivity to local binding of anti-IgG and IgG was similar.

Future experiments will be focused on studying the location and distance-dependent optical properties at the single nanoparticle level by observing the Rayleigh scattering. Also, nanoparticles could be selectively attached to the edges with SERS active conjugated molecules as a linker in order to observe an enhanced SERS signal, since these should be the hot spots.

The following are the overall conclusions from this work.

- Au nanoplates can be synthesized directly on surfaces with improved yield by purification.
- Edge-attached antibodies are more sensitive than the terrace-bound ones since the plasmon fields are the strongest at these sites.
- Controlled binding to edge sites should impact applications in SERS based sensing.
- The RI sensitivity of Au nanoplates could be improved by simply depositing a monolayer of Ag on top of Au.
- The human anti-IgG edge-functionalized Au and Au/Ag nanoplates are stable, highly selective, and highly sensitive to the target protein down to 1 pg/mL concentration. These samples could easily be employed for the detection of more relevant proteins, such as cancer markers.
- The response time of the optical sensor is quite slow and needs improvement for rapid response applications.

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CURRICULUM VITAE

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EDUCATION

University of Louisville, Louisville KY

Ph.D. in Chemistry, August 06 2010

2004-2010

Department of Chemistry (Dr. Francis P. Zamborini)

Dissertation Topic: The Localized Surface Plasmon Resonance (LSPR) Response of Gold and Gold/Silver Metal Nanostructures to Protein Binding and Global Refractive Index Changes.

Kakatiya University, Warangal, India

Bachelors in Pharmacy

2000-2004

RESEARCH EXPERIENCE

University of Louisville, Louisville, KY USA - Fall 2004-present
Synthesized metal nanostructures and used them for Localized Surface Plasmon Resonance (LSPR) Biosensing. This involved seed-mediated synthesis of metal nanostructures directly on surfaces and selective attachment of antibodies on the edges of nanostructures where the Plasmon fields are most intense. We used the nanostructures for antigen detection by monitoring the optical changes by UV-vis spectroscopy. Our goal was to determine the optical detection limit of these protein sensors which is of importance in disease diagnostics and biological studies.

PUBLICATIONS

- 1 Srinivas R. Beeram and Francis P. Zamborini.; Localized Surface Plasmon Resonance Sensing with Ag Coated Au Nanoplates Grown Directly on Surfaces. Manuscript in preparation.
- 2 Srinivas R. Beeram and Francis P. Zamborini.; Sub-Picomolar Label Free Protein Detection with Surface Attached Au Nanoplates by Localized Surface Plasmon Resonance Spectroscopy. Manuscript in Preparation.
- 3 Srinivas R. Beeram and Francis P. Zamborini.; The Effect of Protein Binding Location and Distance on the Localized Surface Plasmon Resonance Response of Purified Au Nanoplates Grown Directly on Surfaces. Manuscript in preparation.
- 4 Srinivas R. Beeram and Francis P. Zamborini.; Purification of Gold Nanoplates Grown Directly on Surfaces for Enhanced Localized Surface Plasmon Resonance Biosensing. *ACS Nano*. **2010**, 4, 3633-3646.
- 5 Srinivas R. Beeram and Francis P. Zamborini.; Selective Attachment of Antibodies to the Edges of Gold Nanostructures for Enhanced Localized Surface Plasmon Resonance Biosensing. *J. Am. Chem. Soc.* **2009**, 131, 11689-11691.

PRESENTATIONS

- 1 **Srinivas R. Beeram** and Francis P. Zamborini. *Purification of Au Nanoplates Grown Directly on Surfaces for Enhanced Localized Surface Plasmon Resonance (LSPR) Biosensing*. **Central Regional Meeting of the American Chemical Society**, Dayton, OH, June 16 - June 19, 2010 – Oral Presentation.
- 2 **Srinivas R. Beeram** and Francis P. Zamborini. *Selective Attachment of Antibodies on Purified Au Nanoplates Grown Directly on Surfaces for Enhanced Localized Surface Plasmon Resonance (LSPR) Biosensing*. **University of Louisville Graduate Research Symposium**, Louisville, KY, March 05 2010 – Poster.
- 3 **Srinivas R. Beeram** and Francis P. Zamborini. *Selective Attachment of Antibodies on Purified Au Nanoplates Grown Directly on Surfaces for Enhanced Localized Surface Plasmon Resonance (LSPR) Biosensing*. **Pittcon**, Orlando, FL, February 28- March 05 2010- Poster.
- 4 **Srinivas R. Beeram** and Francis P. Zamborini. *Selective Attachment of Antibodies on Purified Au Nanoplates Grown Directly on Surfaces for Enhanced Localized Surface Plasmon Resonance (LSPR) Biosensing*.

Electrochemistry- Gordon Research Conference, Ventura, CA, January 10- January 15 2010- Poster.

- 5 **Srinivas R. Beeram** and Francis P. Zamborini. *Selective Attachment of Antibodies on to the Edges of Purified Au Nanoplates Grown Directly on Surfaces for Enhanced Localized Surface Plasmon Resonance (LSPR).* **Electrochemistry- Gordon-Kenan Research Seminar**, Ventura, CA, January 09 - January 10 2010- Oral Presentation.
- 6 **Srinivas R. Beeram** and Francis P. Zamborini. *Selective Attachment of Antibodies on Purified Au Nanoplates Grown Directly on Surfaces for Enhanced Localized Surface Plasmon Resonance (LSPR) Biosensing.* **Electrochemistry- Gordon-Kenan Research Seminar**, Ventura, CA, January 09 – January 10 2010- Poster.
- 7 **Srinivas R. Beeram** and Francis P. Zamborini. *The Effect of Protein Binding Location on the Localized Surface Plasmon Resonance (LSPR) Response of Purified Au Nanoplates Grown Directly on Surfaces.* **36th The Federation of Analytical Chemistry and Spectroscopy Societies**, Louisville, KY, October 2009 – Oral Presentation.
- 8 **Srinivas R. Beeram** and Francis P. Zamborini. *Combining Visible Spectroscopy with Atomic Force Microscopy to Correlate the Optical Properties of Gold Nanoplates with Antibody/Antigen Binding.* **Central Regional Meeting of the American Chemical Society**, Cleveland, OH, May 20 – May 23 2009- Oral Presentation.
- 9 **Srinivas R. Beeram** and Francis P. Zamborini. *Atomic Force Microscopy and Optical Spectroscopy Study of Antibody Attachment to Gold Nanostructures.* **Fifth Kentucky Innovation and Entrepreneurship Conference**, Louisville, KY, April 07 2009- Poster.
- 10 **Srinivas R. Beeram** and Francis P. Zamborini. *Atomic Force Microscopy and Optical Spectroscopy Study of Antibody Attachment to Gold Nanostructures.* **Pittcon**, Chicago, IL, March 07 - March 13, 2009 – Poster.
- 11 **Srinivas R. Beeram** and Francis P. Zamborini. *Atomic Force Microscopy and Optical Spectroscopy Study of Antibody Attachment to Gold Nanostructures.* **University of Louisville Graduate Research Symposium**, Louisville, KY, March 06 2009 – Poster.
- 12 **Srinivas R. Beeram** and Francis P. Zamborini. *Atomic Force Microscopy and Optical Spectroscopy Study of Antibody/Antigen Attachment to Gold Nanostructures.* **KYNANOMAT 2008**, Louisville, KY, March 2008 – Poster.

TEACHING EXPERIENCE

- Graduate Teaching Assistant Fall 2004 – present.
Proctored and graded for General Chemistry 201/202, 101.

Courses Taught:

Introduction to Chemical Analysis I and II – Chemistry 207/208 labs.

Introduction to Chemical Analysis III and IV – Chemistry 209/210 labs.

Organic Chemistry Laboratory I – Chem 343.

My Job involved pre-lab lectures, teaching important concepts and techniques for understanding and performing experiments in the laboratory, setting up chemicals for the lab, pay attention to safety in the laboratory, grading the pre-labs and lab reports.

General Chemistry 201 – Recitations

My job involved answering the questions students had regarding the topics taught by the main instructor and giving quizzes.

Stock Room Assistance: Summer 2009

My job involved preparing chemicals for Organic Chemistry I (343) and Organic Chemistry II (344) labs.

- Undergraduate Research Mentor: Summer 2007 and 2008
Taught basic laboratory techniques, synthesis of nanostructures and their characterization.
- Graduate Research Mentor: Present
Teaching Basic Laboratory techniques, Synthesis of nanostructures and their characterization.

AWARDS

- Excellence in safety in the Teaching Laboratory.
- Travel Award from Graduate Student Council (GSC) for Pittcon- 2009, Central Regional Meeting of the American Chemical Society- 2009, Central Regional Meeting of the American Chemical Society- 2010.
- Travel award from Arts and Sciences Graduate Student Union (GSU) for Central Regional Meeting of the American Chemical Society- 2010

SERVICE ACTIVITIES

- Judging at the 6th Annual Kentuckiana Undergraduate Research Symposium in Louisville – April 2006

LANGUAGES

English, Telugu, Hindi

INSTRUMENTATION

Microscopic Techniques – Atomic Force Microscopy (AFM), Scanning Tunneling Microscopy (STM), Scanning Electron Microscopy (SEM)

Spectroscopy Techniques – UV-vis, FTIR, Raman, Atomic Absorption Spectroscopy (AAS)

Separation Techniques – GC