Signal enhancement of optical immunosensing by gold nanoparticles

Yuan, Xintong

2015

Yuan, X. (2015). Signal enhancement of optical immunosensing by gold nanoparticles.
Master's thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/65564>

<https://doi.org/10.32657/10356/65564>

NANYANG
TECHNOLOGICAL
UNIVERSITY

**SIGNAL ENHANCEMENT OF OPTICAL
IMMUNOSENSING BY GOLD NANOPARTICLES**

YUAN XINTONG

SCHOOL OF MATERIAL SCIENCE AND ENGINEERING

2015

**SIGNAL ENHANCEMENT OF OPTICAL
IMMUNOSENSING BY GOLD NANOPARTICLES**

YUAN XINTONG

YUAN XINTONG

School of Material Science and Engineering

A thesis submitted to Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Master of Engineering

2015

Abstract

Optical signals, such as surface plasmon resonance (SPR) angle, fluorescence intensity and quantum yield are commonly measured in optical immunosensing when an immunological interaction takes place between analytes and the specific bio-recognizable interface. Extensive researches have been therefore concentrated for decades to develop a more sensitive interface to lower the limit of detection. When the chromophores are in proximity to metal nanoparticles, gold nanoparticles (AuNPs) are capable of amplifying the signals by at least one order of magnitude. The enhancement factor is particularly dependent on the separation between the chromophores and surface of metallic nanoparticles, as well as the distribution of the electromagnetic field surrounding to the metallic nanoparticles. In this study, the fluorescence enhancement of Au-avidin-Texas Red conjugate was studied using multiple crosslinkers with different lengths. It was observed that in the presence of oligo-ethylene-glycol-based (OEG-based) crosslinkers not only was stability of gold nanoparticles against protein adsorption improved, but also fluorescence intensity was enhanced. The AuNPs intentionally capped with oligo ethylene glycol ligand will remain colloidal in high ionic strength up to 2mol/l. The fluorescence intensity was enhanced 3.8-fold when CT(PEG)₇ was sandwiched at the length of 35.5Å while 9-fold, in case that CT(PEG)₁₂ was selected at specific length of 47.8Å in the Au-avidin conjugate. It is predicted that such a multiplexing probe can be applied not only as a complementary substrate in SPR immunosensing but also as a fluorescence enhancer in the conventional fluorescence immunosensing.

Acknowledgement

Firstly, I would like to express my sincere thanks and appreciation to my supervisor, Prof Zhang Hua, Prof Robert Marks, for the continuous support and guidance of my research.

Secondly, I would like to thank Dr. Levi Gheber and Dr. Papper for discussions about the fluorescence knowledge. I am astonished by his deep knowledge in this field and thankful to their invaluable suggestion and countless patience during the whole project.

On this occasion, I would also like to express my gratitude and sincere thanks to Dr. Li Hai for the AFM imaging and sample preparation, to Ms. Li Anran for the FDTD calculation and discussion of electric field distribution, and to Mr. Wu Di for the Gaussian Calculation of lengths of crosslinkers.

Last but not the least; I also want to thank all my colleagues, who are sharing their research life every day. Without their infinite hasten, this report can never be finished. Their partnership colors my research life and makes it more comfortable in NTU.

Content

Abstract	i
Acknowledgement	ii
Content.....	iii
List of Figures	v
I. Introduction	1
1.1 Background.....	1
1.2 Objectives	3
1.3 Organization of the thesis	4
II. Literature Review	5
2.1 Optical Immunosensing.....	5
2.2 Fluorescence Biosensing	7
2.3 Metal enhanced Fluorescence	10
2.4 Metal Nanoparticles in MEF.....	14
2.5 State of the problem.....	17
III. Experimental Procedures.....	20
3.1 Chemicals and Reagents	20
3.2 Surface Modification of AuNPs with crosslinkers	21
3.3 Preparation of AuNP-avidin conjugate.....	23
3.4 Instrument and Characterization.....	25
IV. Results and Discussion	27

4.1	Surface modification and Bioconjugation	27
4.2	Colloidal Stability of AuNPs after conjugation	34
4.3	Optical Characterization of the conjugation process.....	36
4.3.1	UV-Vis Absorption spectra of succinimdy esterification	36
4.3.2	UV-Vis Absorption Spectra of AuNPs and Au-avidin conjugate.....	38
4.4	Morphology study of AuNPs and Au-avidin conjugates	42
4.5	Fluorescence study of Au-avidin conjugate	44
4.5.1	Fluorescence Image of Au-avidin conjugate on solid substrate.....	45
4.5.2	Coverage saturation of Avidin onto AuNPs	47
4.5.3	Fluorescence Enhancement of Au-avidin conjugate	49
4.5.4	Influence of Measurement Configuration.....	54
V.	Conclusion and Outlook.....	57
VI.	Appendix	60
	Reference and Bibliography	64

List of Figures

Figure 1 Jablonski Energy Diagram.....	8
Figure 2 Low and high excitation rate in the absence and presence of metal, respectively	12
Figure 3 Summary work on different nanostructures and spacers employing MEF in the biosensing application	16
Figure 4 Thiol compounds used in the modification of AuNPs.....	21
Figure 5 Reaction schemes for carboxylic acid group and free primary amine groups on the protein surface	23
Figure 6 Schematic illustration of synthesis of Au-avidin conjugates through EDC/sulfo-NHS reaction (not in real scale).....	24
Figure 7 Hydrodynamic diameters of 40nm AuNPs after ligand exchange with CT(PET) ₇ and CT(PEG) ₁₂ at various pHs of SPB	28
Figure 8 Stability of thiol-PEG protected AuNPs in various concentration of NaCl. (Left column (L): CT(PEG) ₁₂ –coated AuNPs; Middle column (M): CT(PEG) ₇ -coated AuNPs; Right column (R): citrate-coated AuNPs	29
Figure 9 Hydrodynamic diameter of AuNPs during Surface Activation in different (MES and SPB) buffer.....	30
Figure 10 Hydrodynamic diameter of AuNPs during surface activation with ionic and nonionic additives	31
Figure 11 Hydrodynamic diameter of Au-avidin conjugate in SPB at various concentration of avidin (upper: CT(PEG) ₇ as crosslinker abbr. CT7; Lower: CT(PEG) ₁₂ as crosslinker, abbr. CT12).....	33
Figure 12 Zeta Potential (upper) and Hydrodynamic Diameters(lower) of AuNPs after modification with different crosslinkers (indicated in legend) and after conjugation of avidin (10ug/ml).....	35

Figure 13	Change of sulfo-NHS and AuNPs absorption peak during the activation; Upper inlet: Evolution of the absorbance of sulfo-NHS at 267.5nm as a function of time (min); Lower inlet: Evolution of Peak Absorbance of sulfo-NHS at 267.5nm after eliminating excess NHS/EDC as a function of time(min) over 10 hours.....	37
Figure 14	Normalized UV-Vis absorbance spectra of AuNPs after modification and conjugation of Avidin with crosslinkers at avidin concentration of 10ug/ml.....	40
Figure 15	Normalized UV-Vis absorbance spectra of AuNPs after modification and conjugation of Avidin with crosslinkers at avidin concentration of 0.4ug/ml.....	40
Figure 16	Normalized UV-Vis absorbance spectra of AuNPs after modification and conjugation of Avidin (10ug/ml) without crosslinker but only PEGSH protected.....	41
Figure 17	AFM Image of Au-avidin conjugate (aggregate)	42
Figure 18	SEM Image of Au-avidin with CT(PEG)₁₂ crosslinker (Top: Magnification of 20k, Bottom: Magnification of 120k)	43
Figure 19	Normalized Fluorescence Excitation and Emission spectra of Texas Red	44
Figure 20	Dark field image (upper) and Fluorescence Image (lower) of Au-avidin with CT(PEG)₁₂ crosslinker (magnification of 100X, NA=0.85).....	45
Figure 21	Emission Intensity of supernatant at 613nm (measured by microplate reader). The concentration of avidin illustrated in the curve were diluted from 2.5ug/ml in serial dilution ratio of 1:1.....	48
Figure 22	Emission spectra of Au-avidin conjugate at concentration of 0.5ug/ml (measured by microplate reader with slit width of Ex/Em 9nm/20nm).....	49
Figure 23	Comparison of normalized enhancement factor of Au-avidin conjugates with different crosslinkers.....	51

Figure 24 A Summarized table of other works on different nanostructure/spacers and their corresponding maximum enhancement factor.	52
Figure 25 Emission spectra of Au-avidin conjugate with different crosslinkers and free avidin at concentration of 0.4ug/ml. Inlet: excitation spectra of the corresponding conjugate.....	54
Figure 26 Comparison of Emission Intensity at 613nm of Au-avidin conjugates sandwiched with differernt crosslinkers	56
Figure 27 Calibration curve of concentration of 40nm AuNPs by Lambert-Beer Law	60
Figure 28 Image of AuNPs solution in the presence/absence of NaCL. Cuvettes containing 1:1 solution of AuNPs and two different buffers: PBS (left) and SPB (right) (a). Change in absorbance of AuNPs vs. time as an indication for AuNPs aggregation in the two solutions (b).....	61
Figure 29 UV-Vis Absorbance Spectra of commercialized crosslinker at concentration of 100umol/l	62
Figure 30 Calibration Curve of fluorescence intensity against the concentration of avidin(mg/ml)	63

I. Introduction

1.1 Background

Food and environmental safety is a serious concern for modern society. Even trace amount of toxicant and pollutants will be extremely harmful to humans. Conventional analytical techniques such as high-performance liquid chromatography (HPLC), liquid chromatography mass spectroscopy (LC-MS) and enzyme-linked immunosorbent assay (ELISA) allow for an accurate and sensitive measurement of analytes, however, they also face limitations on low portability and inability for real-time measurement. Therefore, rapid, facile yet highly sensitive sensing techniques have sprung up to substitute the conventional methods, in the markets of medical diagnostic and environmental monitoring.

Being one of the state-of-the-art analytical devices, biosensors are designed as a compact analytical device, combining a biospecific-recognized surface with a transducer system. The specific binding event over a wide range of concentrations between the analyte of interest and the complementary bio-recognizable surface generates a measurable signal, such as an electrical, optical or acoustic signal, which is detected, recorded by the transducer and then transformed into a readable signal. Thus, the generated electronic or photonic signals, which are correlated to the concentration of the analyte, could simply demonstrate the presence of and quantify the specific analyte^{1,2}.

With emerging nanotechnology, integration of nanomaterials with biosensing systems features the possibility of amplifying detection signals and recognizing multiple analytes³. Furthermore, miniaturized biosensors are now

developed into smaller portable and implantable devices⁴. The nanoparticles decorated with biomolecules have been broadly developed as a promising nano-sensor in food pathogen and environmental monitoring, biomarker targeting and clinic diagnostics. Amongst the numerous nanoparticles synthesized to date, gold nanoparticles (AuNPs) are the most attractive owing to their exceptional spectral properties, simplicity of synthesis and well-studied conjugation chemistry, chemical stability as well as excellent biocompatibility⁵. AuNPs can be applied either as an individual luminescence or fluorescence probe, or an auxiliary rectifier to the optical signal from chromophores. In addition, AuNPs have also extensively involved in the lateral flow immunoassay as a colorimetric indicator and antibody carrier. Such lateral flow immunochromatography assays have been extensively researched and emerged in the market for viruses and infectious diseases⁶. AuNPs are also rich utilized in the localized surface plasmon resonance (LSPR) biosensors owing to the absorbance and scattering nature at particular photon energy⁷.

1.2 Objectives

The aim of this study is to determine the maximum fluorescence enhancement in an Au-OEG-protein-fluorophore conjugates. Through alteration of the spacing distance using crosslinkers (abbrev. as OEG) of different chain lengths, emission can be intensified. As a carrier of fluorophore, a protein layer is involved on the surface of AuNPs. The spacer is a promising yet not well-studied heterogeneous structure, which consists of a covalently bound layer of protein and an intermediate OEG-spacer. The conjugate is durable over storage with minimum detachment of biomolecules. After optimizing the parameters of crosslinker and fluorophore, the conjugate will become a potential label to maximize the fluorescence signal in a wide variety of immunoassays. Such an AuNP-based nanoprobe will not only perform as a “nanosensor substrate” for immunoassay in a microfluidic system, but also is applicable as a label-enhancer to improve the performance of conventional fluorescence immunoassays.

1.3 Organization of the thesis

The main body of this thesis is organized as follows:

Chapter 2 reviews the development of optical immunosensing and the principle of fluorescence detection in the first part. Then it briefly describes the mechanism of metal-enhanced fluorescence and its application in biosensing. Last but not the least, the state of problem in the relevant research is summarized.

Chapter 3 describes the experiment section systematically, including the optimization of the modification and conjugation and characterization, followed by chapter 4 where the preliminary results including the colloidal stability, optical characterization, morphology inspection and fluorescence study of Au-avidin conjugate are discussed sequentially.

Chapter 5 summarizes the current works and discusses possible future studies to improve the systematical study of AuNP-enhanced fluorescence and extend its potential application in biomarker diagnostics.

II. Literature Review

2.1 Optical Immunosensing

An optical immunosensor consists of either an antibody or an antigen immobilized onto an appropriate surface. Upon incubation with a complementary target molecule in the sample, the target analyte binds onto the immobilized immunological component and the consequential complex is interrogated optically. Changes in optical signal are indicative of the amount of target molecules present in the sample. Suitable surfaces include planar optical waveguide, optical fibers, metallicly decorated prisms or diffraction gratings.

The function of optical immunosensors is to produce a reliable measurement of the amount of an antibody, antigen or hapten present in samples by transducing the interaction between antibody and antigen into a readable optical signal. Optical immunosensing falls into two categories, namely label-free or labeling methods. The former relies solely upon the binding between antibody and antigen to modulate the signal being measured whilst the latter depends on the usage of a label to visualize the binding event. The analytes spread over a wide range from small haptens (e.g. E3G, Mw=468Da) up to intact virus particles (e.g. influenza virus, Mw=178,000Da) in the clinical test. Non-clinical analytes such as pesticide (2,4-D, atrazine)⁸, mycotoxins⁹ are also involved and they commonly conjugated with larger protein to improve the sensitivity.

Various methods including total internal reflection spectroscopy (Harrick et al, 1967), fluorescent immunoassay (Kronick et al, 1973), surface plasmon resonance (Nylander et al, 1982) have been introduced to improve the

practicability of immunoassay based on different optical properties after the first true optical immunosensor was developed by Giaever et al in 1973 to detect anti-BSA protein and anti-Hepatitis antibody present in human serum using scattering light. Soon after it has developed robustly into miniaturized devices yet higher sensitivity till present and miniaturization of optical biosensor has become a promising research branch in the field of optical biosensor. Recently, Conde's group has developed a prototype by integrating capillary microfluidics with a microfabricated photodiode array into a palm-size instrumentation based on the principle of chemiluminescence ELISA and the LOD of mouse IgG was down to 2nM^{10} . Dong's group invented an opto-microfluidic immunosensor based on the principle of chemiluminescence enhancement by AuNPs. The ultrasensitive sensor enables to detect 17-beta estradiol as low as 2.5pg/ml^{11} .

2.2 Fluorescence Biosensing

A fluorescence-based device monitors the frequency change of emission, which is stimulated by previous absorption of photons and generation of an excited state dissipated in picoseconds. The signal is commonly recorded as image or spectra by either microscopy or spectroscopy as a manner of excitation/emission spectra, correlation, quantum energy and etc. The detailed process of fluorescence transition can be explained by a classical Jablonski's energy diagram, as shown in Figure 1. Without absorption of photons, the fluorophores are at ease in the ground state (S_0). Upon excitation, the fluorophores immediately absorb photons with varying probabilities and jumps into various vibrational energy states, as enlightened in green lines. It is most likely that the fluorophores will relax to the lowest excited singlet state (S_1) in the process, known as vibrational relaxation within picosecond or less. Many pathways for de-excitation are possible, non-radiative transitions, such as intersystem-crossing and internal conversion. The loss of energy is dissipated as heat and absorbed by neighboring solvent molecules and no light will be emitted during the cycles of excited states. When the excited molecules are relaxed from S_1 to S_0 eventually, accompanied with the emission of a photon, the radiative process is formally known as fluorescence, as depicted in red line.

Each fluorescent molecule has a specific absorption and emission spectrum in which excitation\emission take place. The emission spectrum is located at higher wavelengths (lower energy) due to the energy loss in the excited state (Stokes' rule) and the gap between the two peaks is called Stokes' shift. De-excitation of electron can result in a radiation of light according to Stokes rule. The intensity (i) for each of these transitions is defined as the

amount of quantified energy (such as photons) per unit time and unit volume and it is proportional to the concentration of excited molecules (C_0) and given by a simple chemical kinetics:

$$i = AC_0 \cdot e^{-t/\tau} \quad (1)$$

Where τ is denoted as the free-space lifetime of fluorescent molecules relaxing from excited state (S_1) to ground state (S_0), typically within nanosecond regime.

Figure 1 Jablonski Energy Diagram

Immunofluorescence assays have been applied frequently in cell imaging, on the basis of detection strategy of fluorophore-labeled proteins. In general, three types of fluorescence biosensing are comprehensively studied and employed, namely, direct sensing, indirect sensing and fluorescence energy transfer (FRET). The fluorescence signal could be typically in response to the interaction with the analytes by either intrinsic properties of a specific molecule, or external addition of dye in an indirect manner. Common fluorophores, such

as Rhodamin, AlexaFlour®, FITC, Texas Red are tagged to specific target molecules. Fluorescence energy transfer will occur when two moieties of fluorophore are coupled in the way that the emission frequency of one is partially overlapped with the excitation frequency of the other. The fluorophores with higher excitation energy will stimulate the emission of the complementary one if they are placed within few Angstroms apart. By applying FRET, it is possible to visualize in-situ conformational change and concentration of coupling fluorophores.

Although fluorescence is a dominant technique to investigate the structure and dynamics of biological systems, especially in cell biology, fluorescent emission from analytes, especially where tracing amount is requested from biological assays, is somehow weak and interfered by surrounding environment. For instance, the self-quenching or nanogold quenching of fluorescein always occurs, due to FRET between fluorescein molecules and their surrounding dielectric environment¹². Consequently, the intensity of a labeled protein does not emit significant signal despite that extensive labeling is applied, which led to false results in practical bioassay application. To improve the limit of detection, extensive efforts have been concentrated toward amplification of fluorescent signals, including synthesis of amplifying-fluorescent conjugated polymer(AFP)¹³, surface-plasmon enhanced fluorescence(SEF)¹⁴, fluorescent polymerization¹⁵ and etc.

2.3 Metal enhanced Fluorescence

The study of the impact of metallic surface on fluorescent emission dates back to 1970, when Drexhage from IBM Research Laboratory reported that the decay time of fluorophores could be lengthened by reducing the distance of fluorophore above the silver surface¹⁶. This effect could be explained by the distance-dependent reflection of fluorescence emission from metallic surface in far field. Unexpectedly, the amplified emission is only consistent when the fluorophore is placed significantly in the far-field region. When the inter-distance was below 20nm, the lifetime declined dramatically and the emission was strongly quenched. The energy dissipated in an irradiative manner within the metal. Since then, numerous theoretical and experimental studies were conducted to investigate the interaction between an oscillating dipole and metallic films/particles¹⁷ that supports a surface plasma oscillation¹⁸.

Essentially the radiative decay rate of fluorophores is unchanged unless changing the fluorophore itself, however, the performance of fluorescence is sensitive to its environmental change (pH, polarity, temperature, etc.) which are mostly accounted for the non-radiative decay rate. Metallic colloids, exceptionally, change the radiative decay rate of nearby fluorophores. The fluorescent rate of a coupled fluorophore-metallic-nanostructure model is significantly greater than that of the sole fluorophore. The phenomenon is mostly ascribed to altering the energy of free propagating radiation to localized energy in the near field and vice versa by the antenna-behavioral metal nanostructures¹⁹. Observable fluorescence properties include increased quantum yield, shortened lifetimes, enhanced photostability, and an increase in Forster transfer distances and directional emission. When the metal

nanoparticles are irradiated by the incident light, the incident light will be scattered or absorbed in the near-field of the metal nanoparticles and sequentially excites the fluorophores. Then, the fluorophores will undergo internal transitions including both radiative and non-radiative transition. The spectral properties of fluorophores can be dramatically altered by near-field interactions with the electron clouds present in metals. The radiative transition ends up with the emission of a photon at longer wavelength and relaxing the fluorophores to ground state.

Approaching to the surface of metal nanoparticle in the near field will result in a concentrated incident electric field, and will further amplify the excitation rate of the fluorophores, as illustrated in Figure 2. It could be explained by the excitement of the Surface Plasmon on the metal surface when the wave vector of incident photons coincident with that of Surface Plasmon. The constructive interference of two will confine light near the metal surface, which will generate intense local electromagnetic field and magnify the excitation rate of the fluorophores. However, in proximity up to 5nm, where the particle surface is well approximated to a plane boundary, the fluorescence emission is strongly quenched by metallic surface with d^{-3} dependence²⁰, as explained by damping of the dipole oscillations by the nearby metal. The enhancement will thus decline after a maximum enhancement factor.

Figure 2 Low and high excitation rate in the absence and presence of metal, respectively

Another plasmonic enhancement effect is the association of radiative rate with the quantum yield and lifetime of the fluorophores. The transition rate constant is further categorized into the radiative decay rate (Γ) and the non-radiative decay rates (k_{nr}), and commonly used to express the distribution of energy dissipation pathways. The quantum yield (Q_0) of fluorescence is defined as the ratio between emitted photons and absorbed photons when an isolated fluorophore radiated into a homogeneous medium in the far field and can be formulated in the equation (2). Fluorescence free-space lifetime (τ_0) is counted as time from excitation to emission of the fluorophores and is given as the reciprocal of the rate constant, as described in the equation (3).

As a consequence of the electromagnetic coupling of the fluorophore with the nearby plasmonic nanostructures, the excitation-relaxation cycle and the radiative rates increase. Thus the quantum yield is now modified with an additional radiative rate (Γ_m), effectively enabling fluorophores to radiate faster in the proximity to near-field metallic nanoparticles. Unsurprisingly, the effective quantum yield (Q_m) will increase when the change of nonradiative rate

is assumed negligible.²¹ On the other hand, the lifetime (τ_m) will decrease, as described by equation (6)²².

$$Q_0 = \frac{\Gamma}{\Gamma + k_{nr}} \quad (2)$$

$$\tau_0 = \frac{1}{\Gamma + k_{nr}} \quad (3)$$

$$Q_m = \frac{\Gamma + \Gamma_m}{\Gamma + \Gamma_m + k_{nr}} \quad (4)$$

$$\tau_m = \frac{1}{\Gamma + \Gamma_m + k_{nr}} \quad (5)$$

Such plasmonic effect plays a fundamental role on altering the intrinsic behaviors of dipoles of fluorophore molecules and the spectral density modes completely. In other words, it is plausible to pronounce amplification or quenching of emission because they are competitive against distance. There is reasonable theoretical agreement that the maximum metal-enhanced fluorescence occur when the fluorophore is located about 10nm²³ from the silver island film, but many publications disagree with this conclusion and report experimental values ranging from 0nm up to 60nm using different spacers. It is believed that the distance at which the maximum distance occurs varies with different spacing agents²⁴. In addition, the shape, geometry, material all play a role in changing the enhancement effects.

2.4 Metal Nanoparticles in MEF

Over a century, it has been demonstrated that gold or silver nanoparticles exhibit a wide variety of colors, a feature that is intimately correlated to the LSPR property. The profile of extinction spectra is strongly correlated to intrinsic properties of the colloidal metal nanoparticles including composition, shape and size and can for the spherical particles be described by Mie theory²⁵. In addition, the surrounding medium also significantly influences the SPR peak position. It is worth mentioning that most organic molecules causing the extinction and scattering spectrum to shift to longer wavelengths when binding to nanoparticles²⁶.

As metal nanoparticles are used as probes for nano-scale optical spectroscopy, it becomes necessary to optimize them with respect to specific applications. The variation in the fluorescence enhancement is due to the difference in resonance conditions for each particle arising from size and detailed geometry. The nature of material drives the relation between nanoparticles and fluorophores by dielectric constant, ϵ , which is the sole parameter of fluorescence quenching. It also has a great impact on the excitation and radiation rate. Shape of nanoparticles affects the wavelength of its SPR properties as well as its electrostatic polarizability. The nanoparticles in irregular geometric shapes will generate multi-pole and induce inhomogeneous surface charges. Okamoto's group has reported that gold nanoplates and aggregates increased the dye fluorescence significantly, while gold nanorods with high aspect ratio (AR) showed quenching²⁷. The size effect on MEF is more complicated, since the fluorescence emission will not be enhanced further

but when the nanoparticles grows to a critical size. Beyond the critical size, a decreased fluorescence emission is predicted.

Sergey et al.²⁸ reported numerically a maximal fluorescence enhancement factor over 50 times for 50nm silver nanoparticles. Even though extensive studies have demonstrated that colloidal silver particles offer improved enhancement of fluorescence, their use in practical immunoassays is limited because of lack of chemical stability in physiological environment. Gold nanoparticles are attracting greater attention over silver in many biological applications because of favorable properties including chemical stability and biocompatibility. Therefore, most effort is being put into colloidal-gold-based fluorescence enhancement assays^{29,30}. It has been interestingly found that gold nanorods with different aspect ratio will lead to various enhancement factors of both fluorescence and Raman scattering. For instance, stronger absorption along the longitudinal direction and the shift of the peak to longer wavelength widen the applicability of gold nanorods for near-infrared fluorescence biosensing^{31,32}. Moreover, Choudhury et al.³³ have reported that Ag-Au heterostructural nanocomposites could overcome the respective restriction of the lower enhancement factor from Au and the chemical stability from silver, achieving maximum enhancement of up to 50-fold. A summary table is illustrated below in Figure 3 to indicate the previous works relevant to the exploration of Ag/Au nanostructures in MEF for biosensing applications. It is generally accepted that the designed nanostructures usually come out with a higher fluorescence enhancement than colloidal particles given the fact that hotspots build in the nanostructure are much more tunable and will intensify the in-situ electric field dramatically. The fluorescence intensity can thus be

magnified up to 4×10^6 -fold³¹. The species of spacers are also investigated widely from biological couplers²⁸ to inorganic nanolayers³², the latter of which is more precisely controlled.

Citation	Nanostructure	Spacer	Fluorophore	Enhancement Factor
Acuna et al. [34]	100 nm Au nanoparticles	23 nm gap by DNA origami structures	ATTO647N	117-fold
Fu et al. [35]	Gold nanorod	ssDNA	Cy5	2.2-fold
Kinkhabwala, et al. [36]	Gold bowtie nano-antenna	10 nm spacing	TPQDI	1340-fold
Zhang et al. [37]	30nm Au nanoparticles on glass substrate	10nm silica	Alexa Fluor 555	6-fold
Oates et al. [38]	35nm Ag film	7nm alumina	Rhodamine 6G	100-fold
Gabudean et al. [32]	Gold nanorod	9 ± 2 nm thick SiO ₂	Nile Blue	0.38-fold
Guzatov et al. [28]	Silver spherical nanoparticles	Alternate PDADMAC and PSS layers	FITC	5.4-fold
Akbay et al. [29]	Al nanostructured surface	Alternate PSS and PAH layers	BSA protein fluorescence	8.6 fold
Zhou et al. [30]	disk-coupled dots-on-pillar antenna array(D2PA)	Protein-A	IRDye-800cw	4×10^6 -fold
Zhang et al. [39]	70nm-thick silver island film	dextran	Alexa Fluor 680	8-fold
Choudhury et al. [33]	Ag-Au heterostructural nanocomposites	BSA-biotin-Strapavidin	ATTO655	15 fold
Xie et al. [40]	47 nm Au core-Ag shell nanoparticles	HSA monolayer	FITC	13-fold
Xie et al. [41]	periodical Au triangular nanostructures	BSA-biotin-Strapavidin	Alexa Fluor 790	69-fold
Abel et al. [42]	Ag nanowire	BSA-biotin-Strapavidin	FITC	14.3-fold

Figure 3 Summary work on different nanostructures and spacers employing MEF in the biosensing application

2.5 State of the problem

Comprehensive attention is transferred from Forster-resonance-energy-transfer (FRET)-based to metallic-enhanced fluorescence (MEF) immunosensing because of the relatively small Donor-Acceptor distance value of probes compared to the size of the antibodies and the corresponding antigens. In order to avoid the quenching and reaching maximum-fluorescence, one need to find the optimum distance, determined by the characteristics of nanoparticles and the fluorophore, and control it by using the proper immobilization technique. The effective distance between the fluorophores and the nanoparticles can be divided to two components:

(1) The position of the fluorophores on the protein, which is influenced by the conjugation chemistry of the fluorophores.

(2) The immobilization technique in which the proteins linked to the nanoparticles, affected by the length of the crosslinkers.

Nevertheless the capability of generating a MEF-based nanosensor is still challenging although the mechanism of MEF has been well established over decades. Several issues are still challenging and summarized below:

- i. Nanofabrication techniques, such as E-beam lithography enable fabrication of Au/Ag nanostructure in multiple shapes⁴³. However, it suffers from the high manufacturing cost of equipment and only applicable on solid surfaces. Therefore, the sensor chips are only implantable but not injectable nor biodegradable, as thus, the application is restricted in future in-vivo medical diagnostics. In contrast, wet chemical synthesis is a promising method for mass yet economical fabrication⁴⁴.

- ii. Although fluorescence characterizations and subsequent application are routinely practiced in many laboratories. Reproducibility is quite low when it is applied to various immunoassays, especially when the antigens vary in size⁴⁵. The effect of spacer length has not been systematically studied, therefore it is ambiguous to conclude if the signal is enhanced or quenched. Even though theoretical studies have proven that the optimum separation distance to be around 10nm⁴⁶, the experimental results vary broadly, the spacer's dielectric constant in-between AuNPs and fluorophore is an important factor⁴⁷.
- iii. Various spacers were used to separate the fluorophore and nanoparticles, such as oligonucleotide, electrodeposited polymer, spin-cast polymer, polyelectrolytes, silica shell, etc. Each type of spacers may have some limitations, as the properties and morphologies after surface modification cannot be well controlled quantitatively. For instance, oligonucleotide is subject to high cost and degradation by nuclease and the circumstance becomes more severe in the presence of water. Consequently the bio-conjugated AuNPs cannot be stored in buffer for long periods of time⁴⁸. The separation distance can also be fine adjusted by controlling the synthesis parameters of silica shell, but the morphology will undesirably lead to significant level of protein adsorption⁴⁹. Casting polymer sandwiched in AuNPs and fluorophores will alter the dielectric constant⁵⁰, and the dimensions are very difficult to be precisely controlled down to 10nm⁵¹.
- iv. The theory and application of MEF are remarkably studied on silver as a result of better enhancement. However, silver is not stable and easily

oxidized, as compared with gold, especially in physiological buffering system, hence it is considerably limited in biomedical application.

It is promising to investigate the performance of fluorescence enhancement on Au-OEG-protein-fluorophore conjugates. This model is proposed to employ an OEG spacer, which not only reduces the protein adsorption dramatically but also allows for fine adjustment of spacing distance. The above-mentioned problems could be ultimately overcome. After a systematic study of the performance, it can be applicable in the real detection.

III. Experimental Procedures

3.1 Chemicals and Reagents

Poly(ethylene glycol) methyl ether thiol (5000Da) (PEGSH), O-(2-carboxyethyl)-O'-(2-mercaptoethyl)heptaethylene glycol (CT(PEG)₇), polysorbate 20 (Tween20), MES monohydrate, (3-mercaptopropyl)-trimethoxysilane (MPTMS), sodium phosphate monobasic and sodium phosphate dibasic were products of Sigma-Aldrich. CT(PEG)₁₂, N-ethyl-N-(dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS) were purchased from Pierce Thermo and used without further purification. Avidin, conjugated with Texas Red® was purchased from Life Technologies and aliquot to 50ul per tube at the concentration of 5mg/ml upon received. 40nm gold nanoparticles (AuNPs) capped with citrate were purchased from BBInternational with initial molar concentration of 0.149452nM (0.15nM). MilliQ water (18.2MΩ) was used to prepare all reaction and washing buffers in the experiments. The washing buffer and reaction buffer were prepared in stock and kept in the refrigerator at 4°C for future use. EDC and sulfo-NHS were freshly prepared for each experiment. All buffers were stored in the refrigerator to avoid microbial growth. All chemicals and reagents were equilibrated to room temperature in the desiccator before opening to avoid moisture.

3.2 Surface Modification of AuNPs with crosslinkers

Two thiol-terminated oligo ethylene glycol compounds subject to similar functionality yet different chain lengths were involved as crosslinkers in this research, i.e. CT(PEG)₇ and CT(PEG)₁₂ (abbrev. as CT7 and CT12). The chemical structures of crosslinkers are depicted in Figure 4. PEGSH was serving as nonionic protection layer to resist protein adsorption in the control group, where no covalent binding to protein was involved. Briefly, 0.5ml of each crosslinker solution (0.5mM) was added into 1.5ml of AuNPs solution (0.15nM) and mix vigorously for 30 seconds. In the control group, 0.5ml of PEGSH solution (0.2%v/v) was added into 1.5ml of AuNPs of same concentration. The mixtures were incubated at 4°C overnight (>15hours) without light for ligand exchange and formation of rigid self-assemble monolayer (SAM). Excess thiol were removed by centrifugation at 9000rpm (equivalent to 7800g) for three times. The final stock concentration of AuNPs was calculated to be 0.6nM.

Figure 4 Thiol compounds used in the modification of AuNPs

Several parameters were optimized to prevent unwanted aggregation of AuNPs prior to the conjugation of avidin. The reaction buffer for the preparation of Au-avidin conjugate was selected between two buffering solutions, i.e. Sodium Phosphate Buffer (SPB) (10mM, pH 7.4) vs. 2-(N-morpholino)ethanesulfonic acid buffer (MES) (50mM, pH 5.1). It is notable

that SPB is different from Phosphate Buffer Saline (PBS). It is particularly prepared by monobasic and dibasic sodium phosphate in volume ratio of 28:72 in which no NaCl is added. It was found that the as-received AuNPs will aggregate in PBS due to the presence of NaCl (Image shown in appendix).

3.3 Preparation of Au-avidin conjugates

The formation of Au-OEG-avidin conjugate was carried out either by EDC/Sulfo-NHS conjugation (with crosslinkers) or by electrostatic attraction (without crosslinker). The surface of AuNPs was modified with -COOH group, and allowed to react with the amine group in the protein. After esterification, amide linkage substituted Sulfo-NHS groups onto AuNPs. The reaction is schematically described in Figure 5.

Figure 5 Reaction schemes for carboxylic acid group and free primary amine groups on the protein surface

The full conjugation route is illustrated in Figure 6. Briefly, equal volume of EDC (10mM) and sulfo-NHS (2mM) were pre-mixed in the reaction buffer. 1ml of mixed activation solution was immediately added into 1ml of thiol-modified AuNPs and the activation was allowed to incubate for 1hr in the darkness on the shaker. Superfluous reagents were removed by centrifugation at 9000rpm (7800g) for 3 times with washing buffer and the concentration of modified-AuNPs was determined by UV-Visible absorbance spectroscopy. Subsequently, An aliquot of avidin solution was freshly diluted from stock solution (20ug/ml) in ratio of 1:1 with fresh SPB-Tween20 buffer up to 64-fold. Equal volumes of avidin solutions were added into the AuNPs and the mixture was kept stirring for another 2 hours. Excessive avidins were removed by

centrifugation and re-dispersion thrice. And the final product was re-dispersed in SPB at the original concentration of AuNPs.

Figure 6 Schematic illustration of synthesis of Au-avidin conjugates through EDC/sulfo-NHS reaction (not in real scale)

3.4 Instrument and Characterization

To evaluate the spectral and size change of AuNPs during the modification and conjugation, two technologies were generally adopted. The UV-Vis absorption spectra were recorded using a Shimadzu UV-3600 spectrophotometer at the slit width of 1nm. Zeta potential and hydrodynamic diameters of modified AuNPs were analyzed by Zetasizer Nano (Malvern ZS90).

The morphology of Au-avidin conjugate was characterized by Field Emission Scanning Electron Microscope (FESEM) (JEOL 7600F), Transmission Electron Microscope (TEM) (Zeiss Libra120) and Atomic Force Microscope (AFM) in the tapping mode (Bruker). The accelerating voltage of FESEM and TEM were 1kV and 120kV, respectively.

Fluorescence performance was characterized by two methods. The fluorescence spectra were monitored by a Shimadzu RF-5301 spectrofluorimeter operating at an excitation wavelength of 570nm. The spectrofluorimeter was equipped with 10mm*2mm quartz cuvette and the slit widths of excitation and emission were both 5nm. Alternative characterization of fluorescence was conducted with a Tecan M200 microplate reader at the excitation wavelength of 554nm, and the slit widths of excitation and emission were 9nm and 20nm, respectively. Both fluorescence measurements were taken at room temperature. Additionally, the fluorescence image was analyzed by exposing the Au-avidin conjugate onto MPTMS-treated Si wafer and imaged at magnification of 100X (NA=0.85) with Leica DM2500M fluorescence microscopy.

The samples of Au-Avidin conjugates for AFM characterization were prepared on the MPTMS-treated silicon substrates. The substrates were

prepared as follows: the silicon wafer was first cut into 0.3cm*0.3cm pieces and sonicated in acetone and DI water for 5min each. Then the surface of substrates was cleaned by boiling in piranha solution ($\text{H}_2\text{O}_2:\text{H}_2\text{SO}_4 = 3:7$ in volume ratio) at 120°C for 10min. Afterwards, the substrates were rinsed extensively with water, followed by sonication in absolute ethanol (100%) and DI water for 2min each. The organics-free substrates were dried with nitrogen stream and kept in the desiccators for future use. For silanization, the substrates were incubated into 4% freshly prepared MPTMS (in ethanol) solution and sonicate for 30min at room temperature to generate a layer of silane on the surface. The substrates then were rinsed thoroughly with ethanol and water. The dried substrates were cured for another 30min at 80°C to harden the silanized layer. The final dried products were kept in a carefully closed container.

The Au-avidin solution at certain concentration was drop-cast onto the silanized substrate and incubate overnight (>15hours). Then the substrates were washed with DI water to remove loosely bound conjugates and dried with nitrogen.

IV. Results and Discussion

4.1 Surface modification and Bioconjugation

The AuNPs used in this research were synthesized by the one-step reduction of the HAuCl_4 solution with sodium citrate. The as-purchased AuNPs were characterized by UV-Vis absorbance spectroscopy and TEM to confirm the concentration and the morphology, respectively. To allow uniform distribution of negative charges across the surface of the AuNPs and the ease of the subsequent modification, the capping agent of AuNPs was replaced by CT(PEG)₇ or CT(PEG)₁₂ or PEGSH⁵². As the number of ethylene glycol repeating unit is controllable in the crosslinker, protein can be conjugated in an estimated distance at the surface of AuNPs.

PEGSH was selected as control not only to minimize the non-specific binding of avidin, but also to stabilize the dispersion of AuNPs. PEGSH has terminus of thiol group, which facilitates the pseudocovalent gold-sulfur bond formation. The oligo ethylene glycol layer and $-\text{CH}_3$ end provide steric stabilization over aggregation of AuNPs. Additionally, the protective layer will contribute to the resistance to protein adsorption⁵³. Other than PEGSH, Tween20 was employed to replace PEGSH in the negative control during the optimization of conjugate route. As a non-ionic surfactant composed of a hydrophilic head group and a dodecyl tail, Tween20 molecules will form a high-density SAM on a hydrophobic surface which is repulsive against non-specific protein adsorption⁵⁴.

The stability of 40nm AuNPs in range of pH from mildly acidic to mildly basic were evaluated by dispersing modified-AuNPs solution into equal

volume of SPB. In general, the pH of SPB was adjusted to 6.0, 7.0 and 8.0, respectively. As illustrated in Figure 7, the hydrodynamic diameters of AuNPs remained around 45nm. It is clearly observed that AuNPs were stable over pH 6-8 and there was no sign of aggregation at the stage of ligand exchange.

Figure 7 Hydrodynamic diameters of 40nm AuNPs after ligand exchange with CT(PET)₇ and CT(PEG)₁₂ at various pHs of SPB

The SAMs of thiol-PEG will not only increase the size of nanoparticle, they also stabilize AuNPs in saline. The stability was tested by adding NaCl perature. As illustrated in Figure 8, CT(PEG)₇-coated-AuNPs will not aggregate until the concentration of NaCl was increased up to 2M while the CT(PEG)₁₂-coated-AuNPs remained colloidal. In the control group, the citrated-coated AuNPs can only preserve dispersive at much lower concentration of 30mM. In general, it is predictable that thiol-PEG coated AuNPs are applicable not only in high ionic strength of saline, but also in a wide range of pH for further modifications with proteins.

Figure 8 Stability of thiol-PEG protected AuNPs in various concentration of NaCl. (Left column (L): CT(PEG)₁₂ -coated AuNPs; Middle column (M): CT(PEG)₇-coated AuNPs; Right column (R): citrate-coated AuNPs

It is not desirable to observe any aggregation of AuNPs prior to conjugation with avidin. The composition of buffer makes a great impact on the activation. In order to achieve the highest efficiency of conjugation, two kinds of buffer, sodium phosphate buffer was compared against MES buffer at the same pH. The hydrodynamic diameters of AuNPs during the activation can indicate the performance of the buffer. As illustrated in Figure 9, the hydrodynamic diameters increased dramatically in both buffers at the same pH level, indicating that AuNPs coated with either CT(PEG)₇ or CT(PEG)₁₂ aggregated. The aggregation was especially pronounced for the short crosslinker in MES buffer. It is concluded that regardless of the composition of buffers, the carboxylic acid groups failed to activate particularly under acidic conditions and AuNPs could not form a homogeneous dispersion at such conditions.

Figure 9 Hydrodynamic diameter of AuNPs during Surface Activation in different (MES and SPB) buffer

In order to find suitable buffer conditions of activation in MES buffer, the effect of ionic strength was explored by monitoring the size evolution in different MES buffers. MES buffer with high ionic strength was prepared by adding NaCl into 50mM MES buffer until the concentration of NaCl reached 1mol/L, while nonionic MES buffer was prepared by mixing 0.2%v/v Tween20 into 50mM MES buffer. After vigorous mixing the activation solution with AuNPs, 100ul of 1M NaOH was added into the mixture, and the incubation was allowed to equilibrate for 30min. It was found that the aggregate disappeared immediately upon the addition of NaOH. The hydrodynamic diameter was measured and results were summarized in Figure 10. It is obvious that neither salt-containing buffer nor surfactant-containing buffer functioned in preventing AuNPs from aggregation in MES-based buffer.

Figure 10 Hydrodynamic diameter of AuNPs during surface activation with ionic and nonionic additives

Though it is widely reported that MES buffer is suitable as reaction buffer during the activation, it is not applicable in our circumstance⁵⁵. The aggregation was observed under acidic condition regardless of ionic strength of buffer, therefore neutral or basic environment was selected for the activation

step. MES buffer thus was replaced by SPB since the working pH is beyond the buffering range of the former.

A simplified protocol of activation was implemented by substituting Texas Red® conjugated avidin into pure avidin in the conjugation while the pH of activation buffer is basic. Prior to conjugation, no apparent color change and aggregation was observed at pH 8. In the conjugation step, various concentrations of avidin were added into equal volume of AuNPs and the conjugation was incubated in SPB (pH=7.4) for two hours in the shaker. The activated AuNPs were washed with SPB (pH=7.4) at least twice and redispersed in SPB at original concentration of AuNPs. The hydrodynamic diameters of AuNPs were measured at the same conditions, as illustrated in Figure 11. Higher concentration of avidin led to aggregate formation up to 600nm. The diameter was reduced gradually as the amount of avidin was lessened. When the concentration of avidin was as low as 0.5ug/ml, the diameters were found to be 82nm and 62nm for short and long crosslinkers, respectively, and no aggregation could be seen. Similar trend was inspected in both cases of CT(PEG)₇ and CT(PEG)₁₂. Given the fact of stable diameter of AuNPs upon the attachment of avidin, the efficiency of conjugation was improved. The protocol was validated by changing the activation buffer from acidic MES buffer to mildly basic SPB. 10mM of SPB at pH 8 was ultimately applied as the suitable buffer in the activation step..

Figure 11 Hydrodynamic diameter of Au-avidin conjugate in SPB at various concentration of avidin (upper: CT(PEG)₇ as crosslinker abbr. CT7; Lower: CT(PEG)₁₂ as crosslinker, abbr. CT12)

4.2 Colloidal Stability of AuNPs after conjugation

The colloidal stability was studied by zeta potential while the successful modification and conjugation could be confirmed by the changes of hydrodynamic diameter of AuNPs after each step. The changes of these two parameters after each modification step were monitored simultaneously by Nanosizer, based on the principle of dynamic light scattering (DLS). The results were summarized in Figure 12. Each sample was measured three times and the mean values were displayed. The hydrodynamic diameter of AuNPs after ligand exchange increased from 40nm to 44.5nm and to 45nm for CT(PEG)₇ and CT(PEG)₁₂, respectively. The zeta potential showed an increase in the net charge of AuNPs during the ligand exchange from -33.8mV to -48.6mV and to -48.3mV, respectively. The surface remains negatively charged in SPB and their repulsion prevented aggregation. Both parameters demonstrated that colloidal AuNPs were more stable and could be preserved over weeks after the ligand exchange with CT(PEG)₁₂.

After conjugation of avidin of 10ug/ml, it was assumed that the surface of AuNPs was entirely coated with avidin. The hydrodynamic diameters increased dramatically up to 30-fold, which is mostly attributed to the aggregation of AuNPs after conjugation and the hydration of avidin. Nevertheless, the zeta potential decreased to only half its original value, which might be resulted from the partial ionization of avidin in SPB and unreacted COO⁻ groups.

Figure 12 Zeta Potential (upper) and Hydrodynamic Diameters(lower) of AuNPs after modification with different crosslinkers (indicated in legend) and after conjugation of avidin (10ug/ml).

4.3 Optical Characterization of the Conjugation Process

4.3.1 UV-Vis Absorption spectra of succinimide esterification

NHS esterification is a typical reaction in the bioconjugation given the fact of the reaction between the primary amine ($-\text{NH}_2$) and the carboxylic acid ($-\text{COOH}$) in the presence of carbodiimide (EDC). Coupling efficiency is significantly enhanced in the existence of sulfo-NHS due to the increased aqueous solubility of sulfo-NHS. The activation reaction is most commonly performed at pH 4.7-6.0. However, aggregate was observed under acidic condition during the activation. Therefore, pH remained neutral to mildly alkaline (pH 7.2-7.5) in the reaction with primary amines since hydrolysis of NHS-ester will speed up with increase of pH. Therefore, SPB (10mM, pH 7.4) was selected as both the activation buffer and conjugation buffer to maximize the efficiency of conjugation with avidin and avoid aggregation⁵⁶.

The kinetics of the reaction between sulfo-NHS and EDC in ratio of 1:5 was monitored in the cuvette by the decline of absorbance peak at 268nm, which is corresponding to the transformation of sulfo-NHS to sulfo-NHS ester on AuNPs. The reaction took place for 3.5 hours (upper inlet) until when the quantity of sulfo-NHS became unchanged and the sulfo-NHS-terminated AuNPs remained active over 10 hours without hydrolysis (indicated as lower inlet in Figure 13). Additionally the peaks at 528.5nm associated with the plasmonic absorption of AuNPs did not shift or decline, as an indication to satisfactory colloidal stability.

Figure 13 Change of sulfo-NHS and AuNPs absorption peak during the activation; Upper inlet: Evolution of the absorbance of sulfo-NHS at 267.5nm as a function of time (min); Lower inlet: Evolution of Peak Absorbance of sulfo-NHS at 267.5nm after eliminating excess NHS/EDC as a function of time(min) over 10 hours.

4.3.2 UV-Vis Absorption Spectra of AuNPs and Au-avidin conjugate

The conjugation process was simply monitored by the shift of absorbance peak of AuNPs between 400nm and 750nm. It is suggested that not only the surface modification of AuNPs can be visualized, but also the interaction between AuNPs and avidin can be differentiated from conjugation to the physical adsorption. The maximum absorbance was normalized to 1.0 to compare the band shift after each step. The citrate-coated AuNPs in aqueous suspension exhibited an absorption band at 525nm, which corresponded to the typical standard plasmonic band of gold nanoparticles with a mean diameter of 40nm.

The surface of AuNPs was subject to ligand exchange with different types of crosslinker and the subsequent succinimide esterification. As shown in Figure 14, the absorbance peak was red-shifted from 525nm to 528.5nm and 529nm for CT(PEG)₇ and CT(PEG)₁₂, respectively. After the conjugation of avidin, the absorption peaks were further shifted to 560.2nm. Additionally, the band shift was strongly affected by the concentration of avidin. The peak was shift by up to 30nm when avidin fully covered the AuNPs at avidin concentration of 10ug/ml, whilst only 13nm of peak shift was observed if AuNPs was partially covered at 0.4ug/ml of avidin shown in Figure 15. It is well established that the shift of absorption band is significantly influenced by the local refractive index due to the addition of surfactant molecules⁵⁷ and solvent refractive index⁵⁸. However, a strong red shift to 560nm after conjugation of avidin and the broadening of the band were mostly attributed to the aggregation of AuNPs. In addition, due to the aggregation, the peak

absorbance also reduced by half after thorough removing of avidin (data not shown).

Figure 16 illustrated the absorbance spectra of PEGSH-protected AuNPs. The absorbance peak shifted from 525nm to 530nm after PEGSH was added, and further shifted to 536.4nm upon introduction of 10ug/ml of avidin. Compared with the crosslinker groups (around 30nm red-shift), the peak shift was much less significant. As no particular aggregation or color change was observed during the measurement, the peak shift could be explained as the change of local refractive index from the partial coverage of avidin since only physical adsorption dominates in the reaction^{59,60}. It is notable that the absorption band of PEGSH-capped AuNPs performed slight greater red shift than those of CT(PEG)₇ or CT(PEG)₁₂-capped ones after surface modification. The reason could be due to the similar composition yet thick layer of PEGSH.

Figure 14 Normalized UV-Vis absorbance spectra of AuNPs after modification and conjugation of Avidin with crosslinkers at avidin concentration of 10ug/ml.

Figure 15 Normalized UV-Vis absorbance spectra of AuNPs after modification and conjugation of Avidin with crosslinkers at avidin concentration of 0.4ug/ml

Figure 16 Normalized UV-Vis absorbance spectra of AuNPs after modification and conjugation of Avidin (10ug/ml) without crosslinker but only PEGSH protected.

4.4 Morphology study of AuNPs and Au-avidin conjugates

The morphology of Au-avidin conjugates was studied by AFM and FESEM, as depicted in Figure 17 and Figure 18, respectively, where AuNPs (10ug/ml) were conjugated with excess amount of avidin by CT(PEG)₁₂. As shown in the AFM image (Figure 17), the size of individual AuNP was clearly observed yet the size did not significantly change (42.5 ± 3.7 nm) after conjugation as the theoretically estimated size can increase up to 50 nm. It was conspicuously observed in SEM that AuNPs aggregated after conjugation with excess amount of avidin. Avidin has more than one active amine groups, which were reactive to NHS-ester. As a consequence of “bridging effect” of avidin, AuNPs flocculated into clusters.

Figure 17 AFM Image of Au-avidin conjugate (aggregate)

Figure 18 SEM Image of Au-avidin with CT(PEG)₁₂ crosslinker (Top: Magnification of 20k, Bottom: Magnification of 120k)

4.5 Fluorescence study of Au-avidin conjugate

Texas Red is widely used in fluorescence microscopy for dye labeling and cell imaging⁶¹. The excitation and emission spectra are illustrated in Figure 19, where the peaks appear at 595nm and 613nm, respectively.

Figure 19 Normalized Fluorescence Excitation and Emission spectra of Texas Red

The preparation of protein-Texas Red conjugates is accomplished by succinimidyl esterification with biomolecules in a more reproducible manner. The molar ratio of Texas Red to avidin is 3 to 1. At this ratio is the fluorescence yield the highest and the bioactivity of the labeled protein remains high after conjugation⁶².

Two instruments were involved in the fluorescence study. In each measurement, the configuration was setup identically, allowing a direct comparison among different chain lengths of crosslinkers.

4.5.1 Fluorescence Image of Au-avidin conjugate on solid substrate

The fluorescence emission was qualitatively studied by a simple contrast in fluorescence and dark field microscopy images, which were obtained from the same scanned area and at the same magnification level. The comparison of two modes could be found in Figure 20.

Figure 20 Dark field image (upper) and Fluorescence Image (lower) of Au-avidin with CT(PEG)₁₂ crosslinker (magnification of 100X, NA=0.85)

In the dark-field microscopy, the visible light was scattered from gold aggregates. The aggregates in different sizes displayed different scattering intensity. The greater intensity from larger Au aggregates could be elucidated by Rayleigh scattering of AuNPs according to Mie theory, where the scattering coefficient is strongly dependent on the radius of nanoparticles⁶³. Due to the accumulation of avidin, spots with greater light scattering led to stronger fluorescence emission, but its intensity cannot be determined quantitatively as the amount of avidin was not quantifiable on the aggregates.

4.5.2 Coverage saturation of Avidin onto AuNPs

Several parameters are influencing the ultimate performance of fluorescence emission. It is thus crucial to engineer the solely distance while keeping all other parameters constant. It is relatively tricky to determine the exact amount of fluorophores which are bound to nanoparticles because the emission intensity cannot be employed to the number of fluorophores straightforwardly. A common way to determine the locally concentrated fluorophores near nanoparticles is to re-dissolve the fluorophores in the solution, followed by the measurement of emission intensity of those released fluorophores⁶⁴. Nevertheless, the calibration curve of free avidins enlightened an alternative approach to investigate the amount of conjugated avidins in the buffer. In our hypothesis, a subtractive strategy was adopted to confirm the number of loaded avidin since the fluorescence intensity will enhance or quench as a consequence of distance separation. The emission of final Au-avidin conjugate therefore was not accurate to represent the actual degree of enhancement.

In this scenario, the number of bound avidin is determined by subtracting the total number of avidin by that of avidin remaining in the supernatant after conjugation. By comparing the discrepancy of free avidin left in the buffer, the average loading number, denoted as coverage factor, is thus introduced and defined as molar ratio of avidin to AuNPs in the conjugate at the state of saturated coverage at 613nm. As shown in Figure 21, there is a critical point where avidin molecules were fully bound with only a few remained in the buffer, and the emission intensity was initially at least three times greater than noise. The coverage factor was therefore calculated by dividing the molar

concentration of avidin by that of AuNPs at this critical point. The concentration threshold was found to be around 0.625ug/ml and the coverage factor was calculated to be around 166 and 180 for CT(PEG)₁₂ and CT(PEG)₇, respectively. Since the conjugation is not fully accomplished due to partial hydrolysis of sulfo-NHS, it is predictable that the coverage factors were smaller (black and red) than the theoretical values from calculation.

On the other hand, it is obviously inspected that PEGSH-coated AuNPs would conjugate with less avidin. The unbound avidin molecules were left much more in the supernatant since the physical adsorption was much weaker interaction than covalent bond. As a result, lower concentration threshold was found to be 0.125ug/ml in the green curve, and the corresponding coverage factor was calculated to be 40.

Figure 21 Emission Intensity of supernatant at 613nm (measured by microplate reader). The concentration of avidin illustrated in the curve were diluted from 2.5ug/ml in serial dilution ratio of 1:1

4.5.3 Fluorescence Enhancement of Au-avidin conjugate

According to the radiative plasmonic model, 40nm AuNPs are expected to enhance the fluorescence radiation decay rate if the emission peak of the fluorophores locates at longer wavelengths than plasmonic band of AuNPs⁶⁵. It is consistent that emission of Texas Red is red shifted compared to plasmonic band of 40nm AuNPs. Therefore the prerequisite of fluorescence enhancement was fulfilled and an enhancement should be predicted. As illustrated in Figure 22, the emission spectra of different crosslinkers revealed that both two crosslinkers led to the enhanced fluorescence emission intensities compared with free avidin of the same concentration. Au-avidin conjugates sandwiched with longer crosslinker resulted in greater emission intensity.

Figure 22 Emission spectra of Au-avidin conjugate at concentration of 0.5ug/ml (measured by microplate reader with slit width of Ex/Em 9nm/20nm)

In general, there were two effects that enhance the total fluorescence signal concurrently. Firstly, it can be elucidated by the distance dependence on the fluorescence. The spacer to separate the fluorophores from surfaces of AuNPs included a layer of densely-packed OEG and a protein layer atop. Based on the calculation by Gaussian View, the length of CT(PEG)₇ and CT(PEG)₁₂ were 3.55nm and 4.78nm, respectively. The results shown were consistent with the prediction that fluorescence intensity will be enhanced when fluorophores are brought in close proximity to AuNPs within 10nm²³. Secondly, the conjugates are present in form of aggregates in the buffer; therefore some avidins are embedded between AuNPs due to the bridging effect. The gap in between AuNPs in the aggregates generated electromagnetic hot spots and the electric field were therefore significantly amplified. Both of effects could be explained by the coupling of the excited states of the fluorophores to the surface plasmons of AuNPs. As a result, the excitation rate of fluorophores is increased and the apparent intrinsic decay rate is enhanced in the Au-avidin conjugates.

To investigate the effect of AuNPs on fluorescence emission of the homogeneous solution, the fluorescence enhancement was studied by scanning the emission spectra from 580nm to 750nm at avidin concentration of 0.5ug/ml and normalized concentration of AuNPs. At such circumstance, an average quantity of avidin was calculated to be around 40/AuNP, which is smaller than the coverage factor. All the avidin molecules were uptaken and bound to AuNPs thereof. The enhancement factor was defined dimensionlessly as the ratio of emission intensity of Au-avidin conjugate to that of free avidin in solution. By normalizing to the emission intensities of free avidin at 0.5ug/ml at 613nm, the enhancement factor was calculated as 8.6-fold and 3.8-fold for Au-

CT(PEG)₁₂-Avidin and Au-CT(PEG)₇-Avidin conjugates, respectively, as illustrated in Figure 24. The emission intensity at 613nm is proportional to the concentration of avidin at 0.5ug/ml, which is consistent with equation (1). All other parameters including the pH of buffer, volume, instrument setup, measurement temperature, are identical to ensure that the fluorophores are in essentially the same chemical and physical environment.

Figure 23 Comparison of normalized enhancement factor of Au-avidin conjugates with different crosslinkers.

On the contrary, the fluorescence factor of PEGSH-coated Au-avidin is only 0.46, indicating a quenched emission compared with free avidins. It is remarkable that the enhancement of fluorescence obtained from this method was a cumulative intensity instead of studying single conjugate. The results in our experiment were compared with recent works on colloidal nanoparticles-enhanced fluorescence in the summarized table in Figure 24 where nanoparticles

are varied in shape, spacers are different in materials and excitation of dyes are at different wavelength. It is notable that the enhancement factor is different from each paper but normalized to its own reference. In this regard, it is difficult to compare the absolute value of maximum enhancement factor. However, it is reasonable to obtain a higher enhancement factor with those specific nanostructures. The shape of nanoparticles will affect the fluorescence enhancement since the scattering cross section varies from nanoshell⁶⁷ to nanorod⁴⁰. In addition, more hotspots where the electric field can be significantly intensified will be generated at the corners of nanocube. Silica were also extensively introduced to control the distance between AuNPs and the fluorophores, and the maximum intensity enhancement always occur at longer separation distance above 30nm⁶⁶. It is likely due to the change of chemical environment (refractive index, pH) around dye molecules after release, which does not exist in our experiment.

Citation	Nanostructure	Spacer	Thickness(nm)	Fluorophore	Max. Enhancement Factor
Chen et al. [66]	nanoparticle	SiO ₂	36.6	TAMRA-SE@540nm	9.2
Xu et al. [67]	nanocube	CTAB	N.A.	AlPcS@675nm	150
Cheng et al. [68]	nanoparticle	SiO ₂	34nm	Ag ₂ S@665nm	N.A.
Xie et al. [40]	Au/Ag core/shell	HSA monolayer	4nm	FITC@488nm	13
Bardhan et al. [69]	nanoshell	HSA monolayer	4nm	IR800@782nm	40
Bardhan et al. [69]	nanorod	HSA monolayer	4nm	IR800@782nm	9
Zhang et al. [70]	Ag nanoparticles	avidin-biotin	6nm	Cy5@630nm	10

Figure 24 A Summarized table of relevant works on different nanostructure/spacers and their corresponding maximum enhancement factors.

The possible reason of the small enhancement factor might be due to several reasons. The first is due to the random orientation of Texas Red on the avidin molecules, resulting in shorter distance ($<10\text{nm}$) than the theoretically calculated. The second reason might be due to the smaller fluorescence enhancement by gold than that of silver²⁸. The maximum fluorescent enhancement is calculated to be higher if AuNPs are replaced by silver nanoparticles given the fact of greater scattering efficiency whilst absorption being dominant for AuNPs.⁷¹ The third reason is led by large separation between the extinction peak of 40nm AuNPs and the excitation peak of Texas Red. Greater enhancement could be predicted if the excitation peak of fluorophores lies closer to the extinction peak of 40nm AuNPs or larger AuNPs were employed. In addition, there is loss of samples in the steps of the repeated washing and resuspension practically, resulting in a lower amount of materials than calculated.

4.5.4 Influence of Measurement Configuration

The fluorescence emission was measured by an alternative fluorescence spectroscopic method. The slit width was narrowed to 5nm compared to the previous study. Other than the slit width, the detection methods are different. In the microplate reader, the samples were measured in a black well. The excitation light source and photon detectors were located in parallel. Whereas the signal was collected 90° from the light source and the sample was placed in a transparent cuvette in the fluorescence spectroscopy. Since the configuration and parameters are different in two measurements, the emission intensities are not comparable. However, in each method, the parameters are setup identically. The curves obtained from spectrofluorophotometer were plotted in Figure 25.

Figure 25 Emission spectra of Au-avidin conjugate with different crosslinkers and free avidin at concentration of 0.4ug/ml. Inlet: excitation spectra of the corresponding conjugate

The measurement was taken at coverage factor of 180 where avidin was fully bound to AuNPs. At 613nm where there should be an emission peak, none of the crosslinkers succeeded; instead, only a shoulder was observed in the smooth curves. It failed to conclude the correlation between spacing distance and fluorescence enhancement due to insignificant discrepancies of emission curves between CT(PEG)₇ and CT(PEG)₁₂. However, by comparing the intensity at 613nm (Figure 26), it is inevitable to believe that the presence of crosslinker enhances the fluorescence emission intensity to some extent (double), while PEGSH layer quenches the fluorescence.

The discrepancy in emission profile was possibly due to the polarization dependence of excitation. As described before, the excitation and emission was parallel in the microplate reader and perpendicular in spectrofluorophotometer. The discrepancy of emission intensity dropped abruptly, indicating that the magnitude of the enhancement depended on not only the spacing distance but also the orientation of its dipole moment relative to the metallic surface²⁸.

Figure 26 Comparison of Emission Intensity at 613nm of Au-avidin conjugates sandwiched with different crosslinkers

V. Conclusion and Outlook

By combining the biocompatibility, chemical stability plasmonic properties, and capability to enhance fluorescence in near-field, AuNPs have been utilized as promising multiplexing probes in biosensing and bio-imaging. Owing to the superior plasmonic properties, chromophores will exhibit extraordinary emission performance when approaching the surface of AuNPs within evanescent wave field. Given the fact of stability in both buffer and physiological fluid, AuNPs have been employed in real-detection and diagnostic⁷² if they are properly decorated with specific biomolecules and ligands. Alternatively, once integrated with conventional sensor chips, AuNPs can expectably miniaturize surface plasmon excitation devices for point-of-care diagnostics or other field applications.

In this study, the effect of spacing distance on fluorescence enhancement was fundamentally investigated in an Au-protein-fluorophore model. Avidin, conjugated with Texas Red, was employed as biomolecule carrier of fluorophores. Oligo ethylene glycol (OEG) linkers were carefully selected amongst the length-controllable candidates by virtue of a variety of advantages, i.e. economical, rigid, facile conjugation chemistry. Compared with physical adsorption, it is unprecedented to use rigid crosslinkers to covalently conjugate protein to improve the stability of the conjugate. From the economical perspective, OEG offered more practical approach to reduce the cost compared with DNA-spacer. The distance was varied by introducing different numbers of ethylene glycol units, thus the fluorescence enhancement can be systematically studied.

Two crosslinkers, i.e. CT(PEG)₇ and CT(PEG)₁₂ were involved in this study. Full conjugation route was systematically designed with 40nm of AuNPs. Surface ligands of AuNPs were first exchanged with either of the two crosslinkers, followed by a subsequent activation. pH, composition of buffer and concentration of activation solution were optimized so that aggregates of AuNPs were prevented before conjugation. Au-avidin conjugate were formed via NHS/EDC conjugation chemistry through the interaction between carboxylic acid group exposed on AuNPs and the primary amine group on avidin. Morphology, spectral properties, fluorescence emission and colloidal stability of Au-avidin conjugates were extensively studied via multiple techniques. The extinction spectra showed a peak shift up to 4nm after surface modification. A further red shift of 13nm was observed after conjugation with avidin. Aggregation incurred by excess loading of avidin showed a larger peak shift up to 30nm. The fluorescence enhancement was studied by fluorescence spectroscopy. A minimal fluorescence intensity of supernatant after removing the Au-avidin conjugate was an indication of critical point at which avidin molecules were fully bound to AuNPs and the coverage factors were calculated to be around 166 and 180 for Au-CT(PEG)₇-avidin and Au-CT(PEG)₁₂-avidin, respectively. Below this point, avidins are fully bound to AuNPs and fluorescence emission intensity of conjugates was comparable to that of isolated avidins. It is theoretically revealed that the thickness of OEG and protein layer is consistent with the predicted separation distance to enhance the fluorescence. By evaluating the emission spectra, fluorescence factors were calculated to be 3.8-fold by shorter crosslinkers and 8.6-fold by longer ones.

The experiments with Texas Red® labeled avidin on the surface of AuNPs with mean diameter of 40nm have provided a practical prediction on the performance of metal-enhanced fluorescent. The study is yet to be finished and more crosslinkers and fluorophores can be involved to reach the maximal enhancement in the future work. It is thus suggested that a closer coupling between plasmonic band of AuNPs and the excitation of fluorophore could give rise to greater fluorescence enhancement. It is predicted that coupling to AlexaFluor® 532 (Ex/Em at 532nm/554nm) will lead to a significant enhancement⁷³. Additionally, the radiate decay rate and local orientation of fluorophores will be explored to explain the incentives of enhancement. From the perspectives of application, such model can be further developed by linking AuNPs with fluorophores and antibody substituents in an excitation-plasmon-coupling manner to improve the performance of the conventional fluorescence or chemiluminescence immunosensing. Another promising application is SPR-enhanced fluorescence, in which the model can be incorporated to gold or silver substrate to increase both fluorescence intensity and the SPR angle shift⁷⁴. Yet development of a diagnostic device is beyond this study.

VI. Appendix

The absorbance spectra of diluted AuNPs solutions were measured. Calibration curve for the citrate-capped AuNPs at peak absorbance of 525nm is plotted versus the molar concentration of the solution and linearly fitted ($R=0.99964$) according to Lambert-Beer law. The extinction coefficient was calculated as $6.9266 \times 10^9 \text{M}^{-1}\text{cm}^{-1}$ from the slope, which is consistent with the literature⁷⁵. The absorbance responses linearly to concentration of AuNPs above 0.00466nM, The concentrations of subsequent AuNPs after ligand exchange, activation and conjugation were also determined based on this calibration curve.

Figure 27 Calibration curve of concentration of 40nm AuNPs by Lambert-Beer Law

In order to maintain the stability of AuNPs over the experiments, PBS buffer is discarded due to the constituent of NaCl. Absorbance peak of AuNPs at 532nm was recorded over 200 seconds to monitor the aggregation of AuNPs

in Figure 28(b). The color change of AuNPs colloidal solution also demonstrated the aggregation as shown in Figure 28(a).

Figure 28 Image of AuNPs solution in the presence/absence of NaCL. Cuvettes containing 1:1 solution of AuNPs and two different buffers: PBS (left) and SPB (right) (a). Change in absorbance of AuNPs vs. time as an indication for AuNPs aggregation in the two solutions (b).

The absorbance spectra of three types of commercial available NHS-terminated crosslinkers were examined and the curves were plotted in Figure 29. The shoulder appeared between 260 and 280nm, which is consistent with the

absorbance of NHS ester⁷⁶. It is reasonable to determine the extent of formation of NHS-ester in an overturned manner by monitoring the diminishing of peak at around 268nm.

Figure 29 UV-Vis Absorbance Spectra of commercialized crosslinker at concentration of 100umol/l

A calibration curve of fluorescence intensity was plotted as a function of concentration of avidin in Figure 30. The pre-determination guaranteed the functionality of avidin prior to conjugation. In addition, the linear correlation ($R^2=0.9976$) has demonstrated the feasibility that the numbers of unbound avidin remaining in the supernatant could be determined by the measurement of corresponding fluorescence intensity within the range of concentration applied.

Figure 30 Calibration Curve of fluorescence intensity against the concentration of avidin(mg/ml)

Reference and Bibliography

- [1] Velasco-Garcia, M. N. (2009). "Optical biosensors for probing at the cellular level: A review of recent progress and future prospects." Seminars in Cell & Developmental Biology **20**(1): 27-33.
- [2] Eltzov, E., S. Cosnier and R. S. Marks (2011). "Biosensors based on combined optical and electrochemical transduction for molecular diagnostics." Expert Review of Molecular Diagnostics **11**(5): 533-546.
- [3] Venkataramasubramani, M. and L. Tang (2009). Development of Gold Nanorod Lateral Flow Test for Quantitative Multi-analyte Detection. 25th Southern Biomedical Engineering Conference 2009, 15 – 17 May 2009, Miami, Florida, USA. A. McGoron, C.-Z. Li and W.-C. Lin, Springer Berlin Heidelberg. **24**: 199-202.
- [4] Katz, E. and I. Willner (2004). "Integrated nanoparticle-biomolecule hybrid systems: synthesis, properties, and applications." Angew Chem Int Ed Engl **43**(45): 6042-6108.
- [5] Prasad, B. L. V., C. M. Sorensen and K. J. Klabunde (2008). "Gold nanoparticle superlattices." Chemical Society Reviews **37**(9): 1871-1883.
- [6] Biagini, R. E., D. L. Sammons, J. P. Smith, B. A. MacKenzie, C. A. F. Striley, J. E. Snawder, S. A. Robertson and C. P. Quinn (2006). "Rapid, Sensitive, and Specific Lateral-Flow Immunochromatographic Device To Measure Anti-Anthrax Protective Antigen Immunoglobulin G in Serum and Whole Blood." Clinical and Vaccine Immunology **13**(5): 541-546.

-
- [7] Minh Hiep, H., T. Endo, K. Kerman, M. Chikae, D.-K. Kim, S. Yamamura, Y. Takamura and E. Tamiya (2007). "A localized surface plasmon resonance based immunosensor for the detection of casein in milk." Science and Technology of Advanced Materials **8**(4): 331-338.
- [8] Mallat, E., D. Barceló, C. Barzen, G. Gauglitz and R. Abuknesha (2001). "Immunosensors for pesticide determination in natural waters." TrAC Trends in Analytical Chemistry **20**(3): 124-132.
- [9] Maragos, C. M. and M. Busman (2010). "Rapid and advanced tools for mycotoxin analysis: a review." Food Additives & Contaminants: Part A **27**(5): 688-700.
- [10] Novo, P., V. Chu and J. P. Conde (2014). "Integrated optical detection of autonomous capillary microfluidic immunoassays: a hand-held point-of-care prototype." Biosensors and Bioelectronics **57**(0): 284-291.
- [11] Pires, N. M. M. and T. Dong (2014). "Ultrasensitive opto-microfluidic immunosensor integrating gold nanoparticle-enhanced chemiluminescence and highly stable organic photodetector." Journal of Biomedical Optics **19**(3): 030504-030504.
- [12] Harris, B. C., V. Korampally, C. Weilbaecher, L. Polo-Parada, S. Grant and S. Gangopadhyay (2013). "Protease biosensing on novel high surface area organosilicate nanoporous films." Sensors and Actuators B: Chemical **176**(0): 351-359.
- [13] Thomas, S. W., G. D. Joly and T. M. Swager (2007). "Chemical Sensors Based on Amplifying Fluorescent Conjugated Polymers." Chemical Reviews **107**(4): 1339-1386.

-
- [14] Stranik, O., H. M. McEvoy, C. McDonagh and B. D. MacCraith (2005). "Plasmonic enhancement of fluorescence for sensor applications." Sensors and Actuators B-Chemical **107**(1): 148-153.
- [15] Aven, H. J. and C. N. Bowman (2010). "Development of fluorescent polymerization-based signal amplification for sensitive and non-enzymatic biodetection in antibody microarrays." Acta Biomaterialia **6**(1): 83-89.
- [16] Drexhage, K. H. (1969). "Influence of a dielectric interface on fluorescence decay time." Bulletin of the American Physical Society **14**(8): 873-.
- [17] Lakowicz, J. R. (2001). "Radiative decay engineering: biophysical and biomedical applications." Analytical biochemistry **298**(1): 1-24.
- [18] Bohren, C. F. and D. R. Huffman (2007). Absorption and Scattering by a Sphere. Absorption and Scattering of Light by Small Particles, Wiley-VCH Verlag GmbH: 82-129.
- [19] Anger, P., Bharadwaj, P., and Novotny, L. (2006). "Enhancement and Quenching of Single-Molecule Fluorescence." Physical Review Letters, **96**(11), 3-6.
- [20] Campion, A., A. R. Gallo, C. B. Harris, H. J. Robota and P. M. Whitmore (1980). "Electronic energy transfer to metal surfaces: a test of classical image dipole theory at short distances." Chemical Physics Letters **73**(3): 447-450.
- [21] Lakowicz, J. R., C. D. Geddes, I. Gryczynski, J. Malicka, Z. Gryczynski, K. Aslan, J. Lukomska, E. Matveeva, J. Zhang, R. Badugu and J. Huang (2004). "Advances in surface-enhanced fluorescence." Journal of fluorescence **14**(4): 425-441.

-
- [22] Anatoliy I. Dragan and Chris D. Geddes (2012). "Metal-enhanced fluorescence: The role of quantum yield, Q_0 , in enhanced fluorescence" Applied. Physical. Letter **100**(9): 093115
- [23] Sokolov, K., G. Chumanov and T. M. Cotton (1998). "Enhancement of molecular fluorescence near the surface of colloidal metal films." Analytical chemistry **70**(18): 3898-3905.
- [24] Biagini, R. E., D. L. Sammons, J. P. Smith, B. A. MacKenzie, C. A. F. Striley, J. E. Snawder, S. A. Robertson and C. P. Quinn (2006). "Rapid, Sensitive, and Specific Lateral-Flow Immunochromatographic Device To Measure Anti-Anthrax Protective Antigen Immunoglobulin G in Serum and Whole Blood." Clinical and Vaccine Immunology **13**(5): 541-546.
- [25] Bohren, C.G., Huffman, D. R. (2010). "Absorption and scattering of light by small particles." New York: Wiley-Interscience. ISBN 3-527-40664-6.
- [26] Anker, J. N., Hall, W. P., Nilam, C., Zhao, J. and Van, P. (2008). "Biosensing with plasmonic nanosensors." Group, **7**(June), 8–10.
- [27] Horimoto, N. N., Imura, K., and Okamoto H. (2008). "Dye fluorescence enhancement and quenching by gold nanoparticles: Direct near-field microscopic observation of shape dependence." Chemical Physics Letters **467**(1-3), 105–109.
- [28] Guzatov, D. V., S. V. Vaschenko, V. V. Stankevich, A. Y. Lunevich, Y. F. Glukhov and S. V. Gaponenko (2012). "Plasmonic Enhancement of Molecular Fluorescence near Silver Nanoparticles: Theory, Modeling, and Experiment." The Journal of Physical Chemistry C **116**(19): 10723-10733.
- [29] Akbay, N., J. R. Lakowicz and K. Ray (2012). "Distance-Dependent Metal-Enhanced Intrinsic Fluorescence of Proteins Using Polyelectrolyte

Layer-by-Layer Assembly and Aluminum Nanoparticles." Journal of Physical Chemistry C **116**(19): 10766-10773.

[30] Zhou, L. C., F. Ding, H. Chen, W. Ding, W. H. Zhang and S. Y. Chou (2012). "Enhancement of Immunoassay's Fluorescence and Detection Sensitivity Using Three-Dimensional Plasmonic Nano-Antenna-Dots Array." Analytical chemistry **84**(10): 4489-4495.

[31] Lu, X. C., X. Dong, K. Y. Zhang, X. W. Han, X. Fang and Y. Z. Zhang (2013). "A gold nanorods-based fluorescent biosensor for the detection of hepatitis B virus DNA based on fluorescence resonance energy transfer." Analyst **138**(2): 642-650.

[32] Gabudean, A. M., D. Biro and S. Astilean (2012). "Hybrid plasmonic platforms based on silica-encapsulated gold nanorods as effective spectroscopic enhancers for Raman and fluorescence spectroscopy." Nanotechnology **23**(48).

[33] Choudhury, S. D., R. Badugu, K. Ray and J. R. Lakowicz (2012). "Silver-Gold Nanocomposite Substrates for Metal-Enhanced Fluorescence: Ensemble and Single-Molecule Spectroscopic Studies." Journal of Physical Chemistry C **116**(8): 5042-5048.

[34] G. P. Acuna, F. M. Moller, P. Holzmeister, S. Beater, B. Lalkens and P. Tinnefeld, (2012) "Fluorescence Enhancement at Docking Sites of DNA-Directed Self-Assembled Nanoantennas" Science, **338**(6106): 506–510

[35] Fu, Y., J. Zhang and J. R. Lakowicz (2010). "Plasmon-Enhanced Fluorescence from Single Fluorophores End-Linked to Gold Nanorods." Journal of the American Chemical Society **132**(16): 5540-5541.

-
- [36] A. Kinkhabwala, Z. Yu, S. Fan, Y. Avlasevich, K. Müllen and W. E. Moerner, (2009) "Large single-molecule fluorescence enhancements produced by a bowtie nanoantenna" Nature Photonics **3**(11): 654–657
- [37] J. Zhang and J. R. Lakowicz, (2007) "Metal-enhanced fluorescence of an organic fluorophore using gold particles" Optical Express **15**(5): 2598
- [38] Oates T. W. H., Shiratori Y., and Noda S., (2009) "Two-Dimensional Combinatorial Investigation of Raman and Fluorescence Enhancement in Silver and Gold Sandwich Substrates" J. Phys. Chem. C **113**(22):9588–9594
- [39] Zhang J., Fu Y., Liang D., Zhao R. Y. and Lakowicz J. R., (2008) "Enhanced Fluorescence Images for Labeled Cells on Silver Island Films" Langmuir **24**(21): 12452–12457
- [40] Xie F., Baker M.S., and Goldys E. M., (2006) "Homogeneous Silver-Coated Nanoparticle Substrates for Enhanced Fluorescence Detection" J. Phys. Chem. B **110**(46): 23085–23091
- [41] Xie F., Centeno A., Ryan M. P., Riley J. and Alford N. M., (2013) "Au nanostructures by colloidal lithography: from quenching to extensive fluorescence enhancement" J. Mater. Chem. B **1**(4): 536–543
- [42] Abel B., Coskun S., Mohammed M., Williams R., Unalan H. E., and Aslan K. (2015) "Metal-Enhanced Fluorescence from Silver Nanowires with High Aspect Ratio on Glass Slides for Biosensing Applications" J. Phys. Chem. C, **119**(1): 675–684
- [43] Wang, L., W. Xiong, Y. Nishijima, Y. Yokota, K. Ueno, H. Misawa, J. R. Qiu and G. Bi (2011). "Spectral properties of nanoengineered Ag/Au bilayer rods fabricated by electron beam lithography." Applied Optics **50**(28): 5600-5605.

-
- [44] Tabakman, S. M., L. Lau, J. T. Robinson, J. Price, S. P. Sherlock, H. Wang, B. Zhang, Z. Chen, S. Tangsombatvisit, J. A. Jarrell, P. J. Utz and H. Dai (2011). "Plasmonic substrates for multiplexed protein microarrays with femtomolar sensitivity and broad dynamic range." Nature Communication **2**(466): .
- [45] Nooney, R., A. Clifford, X. Leguevel, O. Stranik, C. McDonagh and B. D. Maccraith (2010). "Enhancing the analytical performance of immunoassays that employ metal-enhanced fluorescence." Anal Bioanal Chem **396**(3): 1127-1134.
- [46] Kühn, S., U. Håkanson, L. Rogobete and V. Sandoghdar (2006). "Enhancement of Single-Molecule Fluorescence Using a Gold Nanoparticle as an Optical Nanoantenna." Physical Review Letters **97**(1).
- [47] Lee, S. Y., K. Nakaya, T. Hayashi and M. Hara (2009). "Quantitative study of the gold-enhanced fluorescence of CdSe/ZnS nanocrystals as a function of distance using an AFM probe." Physical Chemistry Chemical Physics **11**(21): 4403-4409.
- [48] Dragan, A. I., E. S. Bishop, J. R. Casas-Finet, R. J. Strouse, J. McGivney, M. A. Schenerman and C. D. Geddes (2012). "Distance Dependence of Metal-Enhanced Fluorescence." Plasmonics **7**(4): 739-744.
- [49] Tovmachenko, O. G., C. Graf, D. J. van den Heuvel, A. van Blaaderen and H. C. Gerritsen (2006). "Fluorescence enhancement by metal-core/silica-shell nanoparticles." Advanced Materials **18**(1): 91-95.
- [50] Matoian, M. A., R. Sweetman, E. C. Hall, S. Albanese and W. B. Euler (2013). "Light Trapping to Amplify Metal Enhanced Fluorescence with Application for Sensing TNT." Journal of fluorescence. **23**(5): 877-880

-
- [51] Tang, F., N. Ma, X. Wang, F. He and L. Li (2011). "Hybrid conjugated polymer-Ag@PNIPAM fluorescent nanoparticles with metal-enhanced fluorescence." Journal of Materials Chemistry **21**(42): 16943-16948.
- [52] Zhang, F., M. W. A. Skoda, R. M. J. Jacobs, S. Zorn, R. A. Martin, C. M. Martin, G. F. Clark, G. Goerigk and F. Schreiber (2007). "Gold nanoparticles decorated with oligo(ethylene glycol) thiols: Protein resistance and colloidal stability." Journal of Physical Chemistry A **111**(49): 12229-12237.
- [53] Shuster, M. J., A. Vaish, M. L. Gilbert, M. Martinez-Rivera, R. M. Nezarati, P. S. Weiss and A. M. Andrews (2011). "Comparison of Oligo(ethylene glycol)alkanethiols versus n-Alkanethiols: Self-Assembly, Insertion, and Functionalization." The Journal of Physical Chemistry C **115**(50): 24778-24787.
- [54] Shen, L., A. Guo and X. Zhu (2011). "Tween surfactants: Adsorption, self-organization, and protein resistance." Surface Science **605**(5–6): 494-499.
- [55] Eck, W., G. Craig, A. Sigdel, G. Ritter, L. J. Old, L. Tang, M. F. Brennan, P. J. Allen and M. D. Mason (2008). "PEGylated Gold Nanoparticles Conjugated to Monoclonal F19 Antibodies as Targeted Labeling Agents for Human Pancreatic Carcinoma Tissue." ACS Nano **2**(11): 2263-2272.
- [56] Bentzen, E.L., et al. (2005). Surface modification to reduce non-specific binding of quantum dots in live cell assays. Bioconjugate Chem **16**:1488-1494.
- [57] G. J. Nusz, S. M. Marinakos, A. C. Curry, A. Dahlin, F. Höök, A. Wax and A. Chilkoti (2008). "Label-Free Plasmonic Detection of Biomolecular Binding by a Single Gold Nanorod" Analytical Chemistry **15**(80): 984-989.

-
- [58] N. Nath and A. Chilkoti (2002). "Label-Free Biosensing by Surface Plasmon Resonance of Nanoparticles on Glass: Optimization of Nanoparticle Size" Analytical Chemistry **76**(18): 5370-5378.
- [59] M. M. Miller, A. A. Lazarides (2005). "Sensitivity of Metal Nanoparticle Surface Plasmon Resonance to the Dielectric Environment" Journal of Physical Chemistry B, **109** (46): 21556–21565.
- [60] Gao, J., X. Huang, H. Liu, F. Zan and J. Ren (2012). "Colloidal Stability of Gold Nanoparticles Modified with Thiol Compounds: Bioconjugation and Application in Cancer Cell Imaging." Langmuir **28**(9): 4464-4471.
- [61] Sandoval, R. M., M. D. Kennedy, P. S. Low and B. A. Molitoris (2004). "Uptake and trafficking of fluorescent conjugates of folic acid in intact kidney determined using intravital two-photon microscopy." American Journal of Physiology-Cell Physiology **287**(2): C517-C526.
- [62] Lefevre, C., H. C. Kang, R. P. Haugland, N. Malekzadeh, S. Arttamangkul and R. P. Haugland (1996). "Texas Red-X and Rhodamine Red-X, New Derivatives of Sulforhodamine 101 and Lissamine Rhodamine B with Improved Labeling and Fluorescence Properties." Bioconjugate Chemistry **7**(4): 482-489.
- [63] Hu, M., J. Y. Chen, Z. Y. Li, L. Au, G. V. Hartland, X. D. Li, M. Marquez and Y. N. Xia (2006). "Gold nanostructures: engineering their plasmonic properties for biomedical applications." Chemical Society Reviews **35**(11): 1084-1094.
- [64] Cheng D., Xu Q-H (2007). "Separation distance dependant fluorescence enhancement of fluorescein isothiocyanate by silver nanoparticles" Chemical Communication **3**(1):248-250.

-
- [65] Guzatov, D. V., S. V. Vaschenko, V. V. Stankevich, A. Y. Lunevich, Y. F. Glukhov and S. V. Gaponenko (2012). "Plasmonic Enhancement of Molecular Fluorescence near Silver Nanoparticles: Theory, Modeling, and Experiment." The Journal of Physical Chemistry C **116**(19): 10723-10733.
- [66] J. Chen, Y. Jin, N. Fahrudin, and J. X. Zhao (2013) "Development of Gold Nanoparticle-Enhanced Fluorescent Nanocomposites" Langmuir, **29** (5): 1584–1591
- [67] Y. K. Xu, S.Hwang, S. Kim, and J. Y. Chen (2014). "Two Orders of Magnitude Fluorescence Enhancement of Aluminum Phthalocyanines by Gold Nanocubes: A Remarkable Improvement for Cancer Cell Imaging and Detection" ACS Appl. Mater. Interfaces, **6** (8): 5619–5628
- [68] H. Cheng, C. Wang, Z. Xu, H. Lin and C. Zhang (2015). "Gold nanoparticle-enhanced near infrared fluorescent nanocomposites for targeted bio-imaging" RSC Advance **5**(1): 20-26
- [69] R. Bardhan, N. K. Grady, J. R. Cole, A. Joshi and N. J. Halas (2009). "Fluorescence Enhancement by Au Nanostructures: Nanoshells and Nanorods" ACS Nano **3** (3):744–752
- [70] Zhang J., Fu Y., Liang D., Zhao R. Y. and Lakowicz J. R. (2009). "Fluorescent Avidin-Bound Silver Particle: A Strategy for Single Target Molecule Detection on a Cell Membrane" Anal. Chem. **81** (3): 883–889
- [71] A. Centeno, F. Xie and N. Alford (2013) "Predicting the fluorescent enhancement rate by gold and silver nanospheres using finite-difference time-domain analysis" IET Nanobiotechnol. **7**(2): 50-58

-
- [72] Xiulan, S., Z. Xiaolian, T. Jian, J. Zhou and F. S. Chu (2005). "Preparation of gold-labeled antibody probe and its use in immunochromatography assay for detection of aflatoxin B1." International Journal of Food Microbiology **99**(2): 185-194.
- [73] Tsurui, H., H. Nishimura, S. Hattori, S. Hirose, K. Okumura and T. Shirai (2000). "Seven-color fluorescence imaging of tissue samples based on Fourier spectroscopy and singular value decomposition." J. Histochem. Cytochem. **48**(5): 653-662.
- [74] Hong, X. and E. A. H. Hall (2012). "Contribution of gold nanoparticles to the signal amplification in surface plasmon resonance." Analyst **137**(20): 4712-4719
- [75] Liu X., Atwater M. et al. (2007). "Extinction coefficient of gold nanoparticles with different sizes and different capping ligands" Colloids and Surfaces B: Biointerfaces **58**(1):3–7
- [76] Miron, T. and M. Wilchek (1982). "A spectrophotometric assay for soluble and immobilized n-hydroxysuccinimide esters." Anal. Biochem. **126**(2): 433-435.