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UNIVERSITY OF CALIFORNIA
RIVERSIDE

Developing Novel Interface and Signal Amplification Strategies for Study of Biological
Interactions by Surface Plasmon Resonance (SPR) and SPR imaging

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Chemistry

by

Ying Liu

September 2012

Dissertation Committee:

Dr. Quan Cheng, Chairperson

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Dr. Yinsheng Wang

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The Dissertation of Ying Liu is approved:

Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE DISSERTATION

Developing Novel Interface and Signal Amplification Strategies for Study of Biological Interactions by Surface Plasmon Resonance (SPR) and SPR imaging

by

Ying Liu

Doctor of Philosophy, Graduate Program in Chemistry

University of California, Riverside, September 2012

Dr. Quan Cheng, Chairperson

Surface plasmon resonance (SPR) has been widely used as a powerful analytical technique for the study of a broad range of biomolecular interactions. With the capability of real-time detection, SPR allows convenient and nondestructive measurement of analyte concentration and binding kinetics. To improve the performance of SPR biosensing, we have developed a series of novel methods that lead to ultrasensitive detection via signal amplification by coupling inline atom transfer radical polymerization (ATRP) to a biospecific interaction. To adapt this enhancement method to the fragile lipid membrane-based system, initiator functionalized gold nanoparticles (AuNPs) has been used with *in situ* ATRP reaction, which allows sensitive measurement of lipid membrane binding proteins. In addition, conjugated nanoparticles AuNPs and Fe₃O₄ NPs have been employed to enhance protein detection sensitivity with SPR imaging. A novel Au-well microarray, fabricated based on spatial variation of metal thickness, is utilized to eliminate background resonance and provide better performance.

To mimic cell membrane recognition processes, a membrane bilayer has been assembled onto a nanoglassified surface, and a synthesized water-soluble deep cavitand was incorporated into the supported lipid membrane. The incorporated cavitand retains its host properties and real-time analysis of the host:guest properties is carried out by SPR and fluorescence microscopy. To further explore the biomolecule interactions on cell membrane, we have studied the polymer growth at the lipid membrane-water interface using functional guest initiator that triggers an ATRP process. A variety of functional polymers have been grown at the lipid membrane surface, demonstrating different hydrophilicity and protein binding properties.

Mass spectrometry in combination with SPR for comprehensive protein analysis has been explored to build a new technical platform. We have designed a thermoresponsive poly(N-isopropylacrylamide) (PNIPAAm) grafted surface, which demonstrates temperature-mediated hydrophobicity for enrichment of proteins based on hydrophobic interaction. SPR quantitatively measures the binding of proteins and peptides, and the biochip is directly interfaced with matrix assisted laser desorption/ionization (MALDI)-MS to characterize the bound analytes. It provides outstanding results in simple steps of on-plate desalting, offering new tools for effective proteomic analysis.

Table of Contents

Acknowledgements.....	vi
Copyright Acknowledgements.....	vii
ABSTRACT OF THE DISSERTATION.....	viii
Table of Contents.....	x
List of Figures.....	xvii
List of Tables.....	xxiii
CHAPTER 1: Introduction.....	1
1.1 Surface Plasmon Resonance (SPR) Biosensor.....	3
1.1.1 Surface Plasmon Resonance: Background and Principle.....	3
1.1.2 SPR Spectroscopy: SPR Sensor Based on Angular Modulation.....	8
1.1.3 SPR Imaging: SPR Sensor Based on Intensity Modulation.....	10
1.1.4 Surface Modification and Immobilization Techniques.....	13
1.1.5 Detection Sensitivity Amplification Strategies for SPR and SPR Imaging..	19
1.1.6 Comprehensive Biological Analysis: SPR / Mass Spectrometry Hyphenation.....	21
1.2 SPR Biosensor for Lipid Membrane Interaction.....	25
1.2.1 Cell Membrane and its Components.....	25
1.2.2 Bilayer Vesicles.....	26
1.2.3 Supported Bilayer Lipid Membranes.....	29
1.2.4 Lipid Membrane Receptor Simulation and Related Molecule Identification.....	36
1.3 Atom Transfer Radical Polymerization (ATRP) Reaction.....	37
1.3.1 ATRP Reaction Principle.....	39
1.3.2 Surface Initiated Polymer Growth.....	42
1.3.3 Application of ATRP Reaction in Biological System.....	44

Reference.....	46
CHAPTER 2: Highly Sensitive Detection of Protein Toxins by Surface Plasmon Resonance with Biotinylation-Based Inline ATRP Amplification.....	54
2.1 Introduction.....	54
2.2 Experimental.....	58
2.2.1 Materials.....	58
2.2.2 Synthesis of Biotinylaed ATRP Initiator.....	58
2.2.3 AFM and Optical Microscopy.....	59
2.2.4 SPR Analysis of Protein Toxin and Signal Amplification with ATRP.....	59
2.3 Results and discussion.....	60
2.3.1 Biotinylated Initiator for Protein Detection and In-line ATRP Process.....	60
2.3.2 Comparison of Amplification Effect for Direct CT Attachment, 1 st Step ATRP Amplification, and 2 nd Step ATRP Amplification.....	70
2.3.3 Morphological Characterization of ATRP Polymer Films.....	72
2.3.4 Effectiveness of Surface Passivation on Background Suppression.....	73
2.3.5 Highly Sensitive Detection of Protein Toxins.....	75
2.4 Conclusions.....	78
Reference.....	79
CHAPTER 3: Detection of Membrane-Binding Proteins by Surface Plasmon Resonance with an All-Aqueous Amplification Scheme.....	82
3.1 Introduction.....	82
3.2 Experimental.....	87
3.2.1 Materials.....	87
3.2.2 Preparation of AuNP-Hydroxyl-Biotin.....	88
3.2.3 Preparation of AuNP-Initiator-Biotin.....	89
3.2.4 Preparation of Calcinated Gold Chips.....	89
3.2.5 Vesicle Preparation.....	90

3.2.6 SPR Measurement and Signal Amplification with AuNP-PHEMA.....	90
3.3 Results and Discussion.....	92
3.3.1 Functionalization of AuNP for ATRP Polymer Growth.....	92
3.3.2 Signal Amplification with AuNP and Inline ATRP on Bilayer Membranes.....	95
3.3.3 Comparison of Signal Amplification Effect with Direct CT Binding, AuNP Amplification, and AuNP-PHEMA Polymer Growth Amplification.....	98
3.3.4 CT Quantitative Detection on Lipid Membrane via AuNP-PHEMA SPR Signal Amplification.....	100
3.3.5 Morphological Characterization of AuNP and ATRP Polymer Films.....	102
3.3.6 Detection of Low concentrations of Protein Toxins in Complex Samples.....	104
3.4 Conclusion.....	107
Reference.....	109
CHAPTER 4: High Sensitive Bacterial Protein Toxin Detection on Patterned Microarray by SPR Imaging with Conjugate Nanoparticle Amplification.....	113
4.1 Introduction.....	113
4.2 Experimental.....	117
4.2.1 Materials.....	117
4.2.2 Preparation of AuNP-Hydroxyl-Biotin.....	117
4.2.3 Preparation of Fe ₃ O ₄ NP-Streptavidin.....	118
4.2.4 Microarray Fabrication.....	119
4.2.5 AFM Characterization.....	119
4.2.6 SPR Spectroscopy and SPR Imaging Measurement.....	119
4.2.7 SPR Signal Amplification with AuNP-Hydroxyl-Biotin and Fe ₃ O ₄ NP-Streptavidin for CT Detection.....	121
4.3 Results and Discussion.....	123

4.3.1 SPR Signal Amplification with AuNP-Hydroxyl-Biotin and Fe ₃ O ₄ NP-Streptavidin Binding.....	123
4.3.2 Morphological Characterization of Fe ₃ O ₄ NP-Streptavidin and AuNP-Hydroxyl-Biotin Nanoparticle conjugate.....	128
4.3.3 Highly Sensitive Detection of Protein Toxin.....	130
4.3.4 SPR Imaging Characterization of Au-well Array and Signal Amplification Measurement.....	131
4.4 Conclusion.....	137
Reference.....	139
CHAPTER 5: Protein and Small Molecule Recognition Properties of Deep Cavitands in a Supported Lipid Membrane Determined by Calcination-Enhanced SPR Spectroscopy.143	
5.1 Introduction.....	143
5.2 Experimental.....	147
5.2.1 Instrument.....	147
5.2.2 Synthesis of Cavitands and Guests.....	147
5.2.3 Calcinated Chip Preparation.....	151
5.2.4 Vesicle Preparation.....	152
5.2.5 Fabrication of Cavitand 1 Receptor Layer and Guest Binding Measurement.....	152
5.2.6 Surface Coverage Calculations.....	153
5.2.7 Kinetic Analysis.....	154
5.3 Results and Discussion.....	155
5.3.1 Cavitand Incorporated Lipid Membrane.....	155
5.3.2 Different Guests Bind to Cavitand Incorporated Lipid Membrane and Binding Affinity Determination.....	158
5.3.3 Fluorescent Guest Binding and Fluorescence Microscopy Characterization.....	161
5.3.4 Biotin Guest Binding and Neutravidin Protein Attachment.....	161

5.3.5 Molecular Modeling for Guest Cavitand Lipid Membrane Binding.....	167
5.4 Conclusions.....	169
Reference.....	171
CHAPTER 6: Protein Recognition by a Self-assembled Deep Cavitand on a Gold Substrate.....	173
6.1 Introduction.....	173
6.2 Experimental.....	174
6.2.1 Instrument.....	174
6.2.2 Cavitand Synthesis.....	176
6.2.3 Surface Preparation and Characterization.....	178
6.3 Results and Discussion.....	181
6.3.1 Self-assembly Monolayer of Cavitand on Gold Substrate.....	181
6.3.2 Guest Recognition.....	187
6.3.3 SPR Imaging Analysis of Cavitand Arrays.....	192
6.3.4 Protein Recognition.....	196
6.4 Conclusions.....	198
Reference.....	200
CHAPTER 7: A Membrane-Bound Synthetic Receptor Promotes Growth of a Polymeric Archipelago at the Bilayer-Water Interface.....	202
7.1 Introduction.....	202
7.2 Experimental.....	207
7.2.1 Instrument.....	207
7.2.2 Compound Synthesis.....	208
7.2.3 Calcinated Chip Preparation.....	209
7.2.4 Vesicle Preparation.....	210
7.2.5 Fabrication of Cavitand 1 Receptor Layer, Initiator Binding and ATRP Reaction Measurement.....	210

7.2.6 FRAP Measurement.....	212
7.3 Results and Discussion.....	214
7.3.1 SPR Characterization of Poly(MMA) Grown From Lipid Membrane Surface.....	214
7.3.2 Contact Angle Measurement.....	217
7.3.3 SPR Characterization of Poly(HEMA) Grown From Lipid Membrane Surface.....	217
7.3.4 Functional Polymer Grown From Lipid Membrane Surface.....	222
7.3.5 FRAP Testing of the Mobility of Lipid Membrane Before and After Polymer Growth.....	224
7.4 Conclusion.....	226
Reference.....	228
CHAPTER 8: SPR Detection of Peptides with On-Plate Desalting and Direct MALDI-MS Analysis on a Thermoresponsive Polymer Surface.....	230
8.1 Introduction.....	230
8.2 Experimental.....	235
8.2.1 Materials.....	235
8.2.2 Preparation of Poly(N-isopropylacrylamide) (PNIPAM) Grafted Substrate and SPR Characterization.....	236
8.2.3 Characterization of PNIPAM Grafted Surface.....	237
8.2.4 Sample Preparation.....	238
8.2.5 On-Plate Desalting and Enrichment of Neurotensin and Peptide Digest Sample.....	239
8.2.6 MALDI-TOF MS.....	239
8.3 Results and Discussion.....	241
8.3.1 Surface-Initiated ATRP of PNIPAM from Gold Surface at Different Temperature.....	241
8.3.2 Surface Characterization.....	246

8.3.3 Protein and Peptide Surface Binding and Desalting.....	246
8.3.4 Application for the Analysis of Protein Digests.....	250
8.4 Conclusion.....	253
Reference.....	254
CHAPTER 9: Summary and Future Outlook.....	256
Reference.....	260

List of Figures

Figure 1.1. Kretschmann configuration in SPR depicting the conversion of energy from light waves to surface plasmons via a gold/dielectric interface.....	7
Figure 1.2. SPR sensor in angular configuration with two parallel channels: a) side view and top view, and b) SPR spectroscopy reflectivity curve and sensorgram.....	11
Figure 1.3 The kinetics of an interaction can be determined from the information in a sensorgram.....	12
Figure 1.4. A schematic of a standard SPR imaging setup with a differential image and the relative sensorgrams.....	14
Figure 1.5. Immobilization methods using chemical linkers with self-assembled monolayer and direct attachment of probe to surface using pyrrole-functional biomolecules.....	16
Figure 1.6. Reaction mechanism for a) carboxylic acid activated by EDC/NHS to generate amide compound and b) amine functionalized surface reacted with thiol modified biomolecule with cross linker SSMCC.....	18
Figure 1.7. SPR signal amplification with a) nanoparticle binding and b) enzymatic reaction.....	22
Figure 1.8. Structure of the cell membrane and L- α -Phosphatidylcholine.....	27
Figure 1.9. Surface-confined membrane models: a) solid-supported lipid bilayer; b) polymer-cushioned lipid bilayer; c) hybrid bilayer, consisting of a self-assembled monolayer (e.g. thiols on Au or silanes on glass or silica) and a lipid monolayer; d) tethered lipid bilayer; e) freely suspended lipid bilayer; f-g) supported vesicular layers.....	30
Figure 1.10. Schematic diagram of a solid supported phospholipids bilayer. The membrane is separated from the substrate by a 10-20 Å thick layer of water.....	32
Figure 1.11. Calcinated chip generation with layer by layer assembly of sodium silicate and PAH, and the formation of bilayer lipid membrane on calcinated chip by vesicle fusion.....	33
Figure 1.12. a) A Cartoon representation of guest binding to cavitand incorporated in a PC bilayer membrane. b) structure of cavitand and the conformation of cavitand with the bound guest trimethylammonium salt.....	38

Figure 1.13. Schematic of the reaction mechanism of ATRP.....	41
Figure 1.14. Overview of techniques for producing grafted polymer layers.....	43
Figure 2.1. A cartoon representation of the biotinylated initiator coupled surface and consecutive two steps of in-situ surface ATRP reactions for SPR signal amplification.....	62
Figure 2.2. SPR sensorgrams for the covalently bound neutravidin-biotin initiator surface showing ATRP with HEMA monomers in the absence a) and presence b) of oxygen-suppression reagent ascorbic acid in the catalyst mixture.....	64
Figure 2.3. SPR sensorgrams for a) immunoassay of 2.92×10^{-12} mol/cm ² CT with ATRP amplification and b) the control channel.....	65
Figure 2.4. SPR characterization of the second step ATRP amplification for a) CT immobilized surface and b) the control surface without CT.....	68
Figure 2.5. Comparison of SPR responses for direct CT coupling (▲), after first ATRP amplification (●), and after second ATRP amplification (■).....	69
Figure 2.6. AFM images of the polymer films prepared with immobilization of 2.92×10^{-12} mol/cm ² of CT. a) after the first step ATRP; b) after the second step ATRP; c) the control surface without CT immobilization; and d) shows the microscopic image of the polymer film prepared with 3.61×10^{-12} mol/cm ² of CT after the second step ATRP.....	71
Figure 2.7. SPR angular shift in response to nonspecific adsorption of 0.25 mg/mL neutravidin (blank) and 20% HEMA (grid) on differently passivated Au substrates.....	74
Figure 2.8. SPR response as a function of CT surface coverage after two steps ATRP amplification with biotinylated initiator and neutravidin. Inset displays the response for CT surface coverage lower than 8.35×10^{-13} mol/cm ²	76
Figure 3.1. A cartoon representation of a) the AuNP surface modification with initiator and biotin, and b) <i>in-situ</i> AuNP coupling through biotin/avidin interaction and surface ATRP reaction for SPR signal amplification in CT detection.....	86
Figure 3.2. SPR sensorgrams for a) initiator and biotin modified AuNP binding to surface via biotin/avidin interaction and b) the control channel.....	93
Figure 3.3. Dynamic light scattering measurements of AuNP (■) and AuNP coated with initiator and biotin after ATRP reaction (●).....	96
Figure 3.4. SPR sensorgrams for a) immunoassay of 120 nm CT on the PC/GM1 surface and its amplification with AuNP coupling and <i>in-situ</i> ATRP, and b) control surface in the	

absence of CT.....	97
Figure 3.5. Comparison of SPR response of direct CT binding (blue), AuNP amplification (green), and AuNP/ <i>in-situ</i> ATRP amplification (red) with a serial of CT concentration 5.88×10^{-7} M, 6.31×10^{-9} M, 5.89×10^{-11} M, 6.31×10^{-13} M, 6.31×10^{-15} M, 6.31×10^{-16} M.....	99
Figure 3.6. SPR response curve for CT detection using AuNP/ATRP amplification. Inset is the calibration curve with CT concentration lower than 6.3×10^{-8} M for emphasizing detection of low concentrations.....	101
Figure 3.7. AFM images of a) the surface attached AuNP in the presence of 5.9×10^{-7} M CT and b) after <i>in-situ</i> polymer growth. Bottom are microscopic images of the AuNP-PHEMA films prepared with c) 6.3×10^{-13} M, d) 1.3×10^{-7} M and e) 5.9×10^{-7} M CT after AuNP/ATRP amplification.....	103
Figure 3.8. (Top) Comparison of SPR measurement of 1 μ M, 10 nM, 50 pM CT in PBS buffer and in 50% diluted rabbit serum after AuNP/ATRP amplification. (Bottom) SPR sensorgrams showing immunoassay and amplification on PC/GM1. a) CT-spiked 50% rabbit serum, and b) control with 50% rabbit serum only.....	106
Figure 4.1. Cartoon representation of the fabrication of gold well array via photolithography.....	120
Figure 4.2. Cartoon representation of SPR imaging signal amplification from consecutive different size conjugated nanoparticles binding on gold well array.....	124
Figure 4.3. SPR sensorgrams for a) immunoassay of 2.51×10^{-13} mol/cm ² CT with AuNP-Hydroxyl-Biotin and Fe ₃ O ₄ NP–Streptavidin nanoparticle for signal amplification and b) the control channel in the absence of CT.....	125
Figure 4.4. SPR sensorgrams for Fe ₃ O ₄ NP-Streptavidin nanoparticle binding on covalently immobilized Biotin-BSA in the presence (black) and absence (red) of AuNP-Hydroxyl-Biotin nanoparticle.....	127
Figure 4.5. AFM image of Fe ₃ O ₄ NP-Streptavidin and AuNP-Hydroxyl-Biotin conjugate nanoparticles binding on sensorchip in the presence of 2.51×10^{-13} mol/cm ² CT.....	129
Figure 4.6. SPR response as a function of CT surface coverage after AuNP-Hydroxyl-Biotin and Fe ₃ O ₄ NP-Streptavidin conjugate nanoparticle amplification with streptavidin bridge.....	132
Figure 4.7. Raw SPR images of gold well microarray a) before CT immobilization and b) 1.29×10^{-12} mol/cm ² CT after nanoparticles amplification. c) Difference SPR images of	

1.29x10 ⁻¹² mol/cm ² CT after nanoparticle amplification by subtracting a) from b), and d) before amplification. The corresponding e) 3D and f) 2D intensity profile for the array Elements.....	134
Figure 4.8. SPR images of 2x5 arrayed gold wells with a) control, b) 3.16x10 ⁻¹⁵ mol/cm ² , c) 7.59x10 ⁻¹⁵ mol/cm ² , d) 1.00x10 ⁻¹³ mol/cm ² and e) 1.29x10 ⁻¹² mol/cm ² after Fe ₃ O ₄ -Streptavidin and AuNP-Biotin conjugate nanoparticle amplification.....	136
Figure 5.1. a) Cavitands 1-3; b) minimized conformation of 1 (SPARTAN, AM1 forcefield) with one bound choline molecule (4) in the cavity; c) the guests used in this study.....	148
Figure 5.2. A cartoon representation of guests binding to cavitand 1 incorporated in a PC bilayer membrane.....	157
Figure 5.3. SPR sensorgrams for guest 6 interaction with PC bilayer membrane in the presence (left) and absence (right) of cavitand 1.....	159
Figure 5.4. Fluorescence microscopic images of the calcinated chip containing a) PC bilayer, cavitand 1 and guest 6; b) PC bilayer and guest 6 alone (control).....	163
Figure 5.5. SPR sensorgrams for NeutrAvidin interaction with a supported PC membrane containing a) cavitand:guest complex 1•7; b) cavitand 1 and biotin control; c) derivatized biotin guest 7 in the absence of cavitand.....	165
Figure 5.6. Illustration of the relative sizes of the cavitand 1•guest 6 complex and POPC lipids (individual components minimized by SPARTAN, AM1 forcefield).....	168
Figure 6.1. a) Synthesis of cavitands 1-3; b) Minimized conformation of 1 (SPARTAN, AM1 forcefield) with a NMe ₃ ⁺ -tagged guest in the cavity; and c) the guests used in this study.....	175
Figure 6.2. Illustration of cavitand 3 adsorption on the surface in the presence of a) space-filling molecules MUA or ODT, b) choline chloride and c) no additive.....	183
Figure 6.3. Cyclic voltammetry characterization of various surface coverages; charge carrier 2.5 mM K ₃ Fe(CN) ₆ in 25 mM KCl at 100mV/s.....	185
Figure 6.4. Sensorgrams showing the SPR response upon addition of guest 4 to the representative surfaces.....	189
Figure 6.5. a) SPR imaging of fluorescent guest 4 incubated on a 2x2 array of cavitand 3 (4mm in diameter) and b) corresponding intensity 3D profile.....	195
Figure 6.6. SPR sensorgram of streptavidin incubated with 10:1 MUA:Cavitand 3 SAM	

Au substrate a) in the presence and b) absence of biotinylated guest 5.....	197
Figure 7.1. a) Tetracarboxylate cavitand 1 and the minimized conformation of 1 (SPARTAN, AM1 forcefield) with one bound initiator molecule (2) in the cavity; b) the guests and monomers used in this study; and c) representation of the polymer synthesis and regeneration experiment.....	204
Figure 7.2. Representation of polymer growth at a cavitand-impregnated supported lipid bilayer.....	206
Figure 7.3. SPR sensorgram for cavitand-mediated synthesis of poly(MMA) (above) and control experiment in the absence of cavitand (bottom).....	213
Figure 7.4 SPR sensorgram indicating the living polymer growth by repeating injection of monomer MMA and catalyst.....	215
Figure 7.5. Effect of <i>in situ</i> polymerization on film hydrophobicity. a) POPC supported membrane; b) after growth of poly(MMA) at the cavitand-embedded membrane surface.....	216
Figure 7.6. SPR sensorgram for cavitand-mediated synthesis of poly(HEMA).....	218
Figure 7.7. a) Scheme of reversible polymer growth and b) SPR sensorgram indicating the repeated removal/construction process with poly(HEMA).....	220
Figure 7.8. Repeat ATRP reaction with second injection initiator.....	221
Figure 7.9. Fluorescence microscopy image of fluorescently tagged avidin bound to a poly(biotin-AEMA) polymer grown at the membrane interface and cartoon of the experiment (top). SPR sensorgram demonstrating avidin-fluorescein attached on poly(biotin-AEMA).....	223
Figure 7.10. Confocal microscopy images of the FRAP experiment for a supported lipid bilayer consisting of 5% NBD-labeled PC and 95% PC lipids on a cleaned glass slide. Pristine refers to membrane before photobleaching, and a-e show images obtained 3, 21, 39, 57 and 75 s after bleaching, respectively (above). FRAP recovery fitting curve obtained with a supported lipid bilayer consisting of 5% NBD-labeled PC and 95% PC lipids on a cleaned glass slide (bottom).....	225
Figure 8.1. Cartoon representation of the PNIPAM growth from gold substrate via SI-ATRP reaction and on-plate desalting process based on PNIPAM polymer chain hydrophilicity change.....	240
Figure 8.2. SPR sensorgrams of SI-ATRP PNIPAAm polymer growth from gold substrate a) at room temperature and b) at 60°C.....	242

Figure 8.3. SPR sensorgram of PNIPAAm polymer chain configuration change according to temperature.....	244
Figure 8.4. Contact angle measurement of PNIPAAm grafted surface a) at room temperature and b) 60°C.....	245
Figure 8.5. SPR sensorgrams for the adsorption of Cytochrome c on PNIPAAm at a) 60°C and b) room temperature.....	247
Figure 8.6. SPR sensorgrams for the adsorption of neurotensin on PNIPAAm at a) 60°C and b) room temperature.....	249
Figure 8.7. Mass spectra of 120 pmol neurotensin with 100 mM NaHCO ₃ mixture solution a) before desalting on normal stainless MALDI plate and b) after PNIPAAm on-plate desalting.....	251

List of Tables

Table 5.1. Binding properties of guest molecules 4-8 in the PC•1 complex.....	162
Table 6.1. SPR resonance angle change upon guest binding.....	191
Table 8.1. Mass spectra of 2 pmol α -casein digests with C18 tip desalting and PNIPAAm on-plate desalting.....	252

Chapter 1 Introduction

A complete understanding of biomolecular interactions is of great significance to biology and bioanalytical chemistry, and the study of biomolecular interactions has undergone great progress in recent years largely due to the development of state-of-the-art label-free technologies. The application of label-free technologies is currently at a high level, generating considerable interest in drug discovery, medical diagnostics, and environment monitoring. Affinity measurements, kinetics (on/off rates), and quantitative analysis are some of the examples for the desired label-free analyses. These assays have been proven very valuable for routine analysis involving biomolecular interactions, and the trend is moving towards high-throughput protein analysis for biological characterization and screening. Surface plasmon resonance (SPR) is the best known and most used label-free technology.

SPR is a label-free optical technique¹ that detects with high sensitivity ($<10^{-6}$) and fast time resolution (<1 s) the changes in refractive index of a surface coating or solution near the SPR active surface. Functionalization of the metal surface with specific binding sites allows a bioaffinity interface to be established that can detect molecular interactions in real time and without the need of a label. Therefore, SPR spectroscopy has become a popular tool in biochemistry and bioanalytical chemistry, especially for determining the

on/off rates and equilibrium binding constants for interaction between proteins, DNAs, RNAs, and a wide variety of biomolecules/ligands, and for investigating the effects of cofactors and inhibitors on the binding properties².

My research goal is to develop new SPR methodologies to characterize interactions of biological molecules with enhanced performance. This chapter will first review the fundamental principles and current progress in SPR-based methods. Effort will be placed on several aspects of SPR detection, in particular ultrasensitive detection of proteins. The ability to carry out highly sensitive protein measurements by SPR techniques at low concentration is significant and will be practically very valuable. To improve SPR performance, my work is primarily focused on developing novel signal amplification strategies. These include application of in situ ATRP polymerization, modification of protocols for fragile supported membrane systems, and adaptation of signal amplification for high-throughput protein analysis by SPR imaging. My work also deals with molecular interactions of a synthetic cavitand incorporated in the lipid membrane as receptor mimic, and characterizes the affinity properties of different guest molecules. Lastly my research effort is placed on the development of hyphenated techniques that couple SPR and mass spectroscopy by using thermoresponsive polymer grafted substrates.

1.1 Surface plasmon resonance (SPR) biosensor

1.1.1 Surface plasmon resonance: background and principle

The concept of surface plasmons was originated in 1902 by R. W. Wood³, who first described his observation of unusual diffraction on diffraction gratings. Later in 1941, the phenomenon was attributed to the excitation of surface waves by Fano⁴. Eventually the excitation of surface plasmons in thin metal films penetrated by electrons was theorized and broadly accepted in the 1950s^{5, 6}. The electron energy loss resulted from excitation of a surface plasma oscillation, in which part of the electric field extended beyond the specimen boundary. Therefore, the surface plasma oscillation could be affected by the presence of a film or contaminant on the specimen surface. Otto, Kretschmann and Raether are credited for establishing the foundation and applicability of SPR. In 1968, Otto demonstrated that the drop in the reflectivity in the attenuated total reflection is due to the excitation of surface plasmons⁷. In the same year, Kretschmann and Raether reported excitation of surface plasmons in another configuration using attenuated total reflection⁸. The Kretschmann configuration is especially convenient and is the basis for most SPR instrumentation to this day. However, despite being known as a new optical technique, it was not until the late 1980s when Raether⁹ popularized SPR into a label-free optical technique that is widely employed today. A company, Pharmacia Biosensor AB, was founded in 1984 to develop and market a functional SPR instrument. The

development of appropriate sensor surfaces¹⁰ and the fabrication of the silica microfluidic cartridge¹¹ provided the convenience and simple operation of the SPR instrument.

Surface plasmons (SPs) are surface electromagnetic waves that propagate parallel along a metal/dielectric interface. The electromagnetic theory of SPR is based on solving Maxwell's equations using the modal method¹². SPR occurs when plane-polarized light hits a metal film under total internal reflection (TIR) conditions. When a light beam propagating in a higher refractive index medium reaches the interface with a lower refractive index medium at an incident angle above a critical value, the light is totally reflected at the interface and propagates back into the higher index medium. Although no light is coming out of the prism in TIR, the electrical field of the photons extends into the low index medium¹³. This field is called the evanescent wave because the amplitude of the wave decreases exponentially with increasing distance from the interface surface, decaying over a distance of about one light wavelength¹⁴. The penetration depth of the evanescent field wave is defined as the distance over which the wave decays to $1/e$, or about 37%, of its maximum intensity.

The different polarization of the evanescent field has to be distinguished since they behave differently at the interface. It can be decomposed into a component that is lying

also within the xz-plane and an orthogonal component that runs parallel to the y-axis. The xz-plane component is called p-polarization (TM-mode), and the orthogonal component corresponds to an electric field of s-polarization (TE-mode).

The prism used for SPR excitation is coated with a thin film of a noble metal on the reflection site. In most cases, gold is used because it is chemically inert to solutions and solutes typically used in biochemical contexts. The p-polarized component of the evanescent field wave penetrates into the metal layer and excites electromagnetic surface plasmon waves and creates an enhanced evanescent wave. Therefore, some of the light energy is “lost” into the conductor film, and the surface plasmon angle (θ_{spr}) exists when the loss is greatest and the intensity of reflected light reaches a minimum.

The surface parallel wave vector of incident light, k_x , is a function of incident angle (θ), refractive index (n_1 , higher index medium) and wavelength (λ).

$$k_x = \frac{2\pi}{\lambda} \times n_1 \times \sin(\theta) \quad (1-1)$$

The surface plasmon's wave vector, k_{sp} , is a function of refractive indices of both lower index medium (n_2) and conductor layer (gold, n_{gold}).

$$k_{sp} = \frac{2\pi}{\lambda} \times \sqrt{\frac{n_{gold}^2 \times n_2^2}{n_{gold}^2 + n_2^2}} \quad (1-2)$$

Since k_{sp} is fixed for a given substrate, the surface plasmon resonance condition ($k_{sp} = k_x$) can be satisfied by tuning surface portion of incident light wave vector, k_x . Based on equation 1-1, k_x can be tuned to equate k_{sp} by varying either the incident angle (θ), or the

wavelength (λ). Therefore, SPR measurements are collected in one of two modes: (a) scanning angle and (b) wavelength shift. Scanning angle SPR is the most widely used method. A single-wavelength light source is used for excitation and it measures the refractive index changes from a prism/gold assembly as a function of the incident angle. Surface plasmons are generated at the prism/gold interface at θ_{spr} and the reflected intensity is damped. A minimum of the reflected intensity occurs at the SPR angle.

The Kretschmann configuration is the major setup to excite surface plasmons. This configuration is shown in Figure 1.1, which employs p-polarized light that is totally internally reflected at the metal surface. In this configuration a laser beam or light emitting diode (LED) is reflected off the base of a high refractive index prism and the reflected intensity is measured. A thin metal layer, about 51 nm of gold, is located on the prism base which works as the sensor chip, followed by a bulk dielectric (usually water or buffer). The metal layer thickness needs to be precisely controlled in order to obtain the most efficient coupling to the excitation of the surface plasmon for maximum surface sensitivity.

Biomolecular interactions taking place at the SPR surface change the surface mass and thus the refractive index within the range of the evanescent field wave. The SPR angle is therefore altered and is measured as a response signal. The SPR angle change resulting from biological events are monitored in real time in a continuous manner and forms a

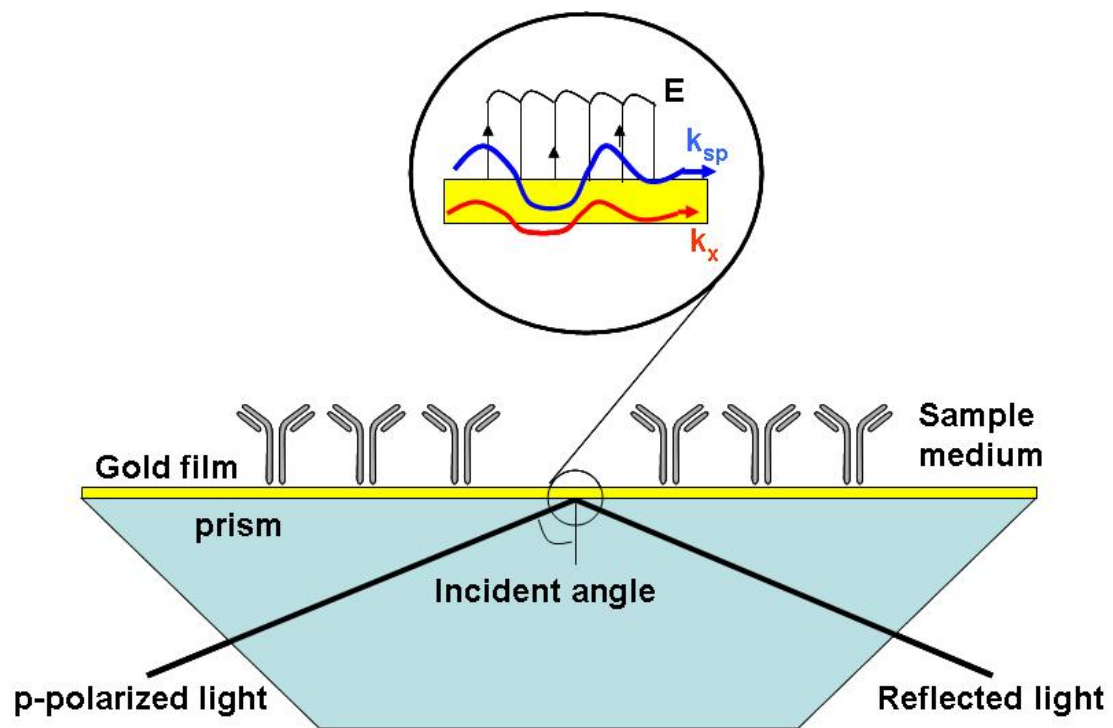


Figure 1.1. Kretschmann configuration in SPR depicting the conversion of energy from light waves to surface plasmons via a gold/dielectric interface.

sensorgram, providing quantitative information on specificity, activity, affinity and kinetics of biomolecular interactions. Therefore, SPR is a powerful surface analytical technique allowing for label-free, nondestructive study of interfacial properties and processes involving both chemical and biological species.

1.1.2 SPR spectroscopy: biosensing based on angular modulation

The phenomenon of surface plasmon resonance occurs at a metal/dielectric interface where one of the biological binding partners is immobilized on a metal surface while the other binding partner is allowed to flow across the sensing interface. SPR spectroscopy monitors the changes in refractive index occurring at the metal surface upon interaction between the two bio-specific ligands. In the early 1990s, an angular modulation-based SPR sensor with a refractive index resolution of about 2×10^{-6} RIU was reported¹¹. The sensor consisted of a light-emitting diode (LED, wavelength – 760 nm), a glass prism and a detector array with imaging optics. A divergent beam produced by the LED was collimated and focused by means of a cylindrical lens to produce a wedge-shaped beam of light that was used to illuminate a thin gold film on the back of a glass prism containing several sensing areas (channels). The detector measured the angular signal change. Figure 1.2a demonstrates the flow cell setup with two channels, therefore two measurements can be carried out in parallel. The bound protein generates SPR signal and produces two different graphs (Figure 1.2b). One is the reflectivity curve, demonstrating

the intensity of reflected light change according to resonance angle shift. The other is the sensorgram, with the resonance angle shift versus time where the surface binding event can be monitored in real time.

The SPR sensorgram generally contains three phases: the association phase, the dissociation phase and the regeneration phase, as shown in Figure 1.3. The binding kinetics that quantitatively characterizes a biomolecular interaction by rate constants and equilibrium constants can be determined from the sensorgram as well.

For an SPR measurement, the reaction rate and equilibrium constants of interactions can be assessed with the reaction:



where A is the biological/chemical analyte and B is the ligand immobilized on the gold sensor surface. The equilibrium dissociation constant can be determined from the sensorgram by:

$$AB_{eq} = AB_{max} \times \frac{1}{1 + \frac{K_D}{[A]}} \quad (1-4)$$

where AB_{eq} is the average of the response signal at equilibrium in defined intervals for each concentration of analyte, $[A]$. The binding association rate and dissociation rate can also be determined from binding kinetic sensorgram¹⁵. Generally, in the initial rate kinetic method, at $t = 0$, the equation for initial rate analysis is:

$$\frac{d_{AB}}{d_t} = AB_{\max} \times [A] \times k_{ass} \quad (1-5)$$

where $AB_{\max}$ is the maximum response that can be obtained for analyte binding on the sensor surface, and k_{ass} is the association constant in units of $\text{mol}^{-1}\text{s}^{-1}$. By plotting the initial rate against analyte concentration A , a straight line is obtained with a slope equal to $AB_{\max} \times k_{ass}$. After k_{ass} is determined, k_{diss} , the dissociation constant, in units of s^{-1} for an AB-type reaction, can be determined by the equation:

$$AB_t = (AB_0 - AB_{\infty}) \times e^{-k_{diss} \times t} + AB_{\infty} \quad (1-6)$$

where AB_0 is the initial response (i.e. the beginning of the dissociation curve), and AB_{∞} is the final response once completely dissociated. The association and dissociation constants represent the affinities of interaction rather than kinetic constant values. Briefly, the value for the equilibrium dissociation constant, K_D , can be determined from the rate constants by the following:

$$K_D = \frac{k_{diss}}{k_{ass}} \quad (1-7)$$

1.1.3 SPR imaging: SPR sensor based on intensity modulation

While highly useful for determining a variety of important kinetic and affinity parameters for biological interactions, one major drawback of SPR is its low-throughput nature. This problem has been largely circumvented with the advent of SPR imaging or SPRi technique. SPRi couples the sensitivity of scanning angle SPR measurements with

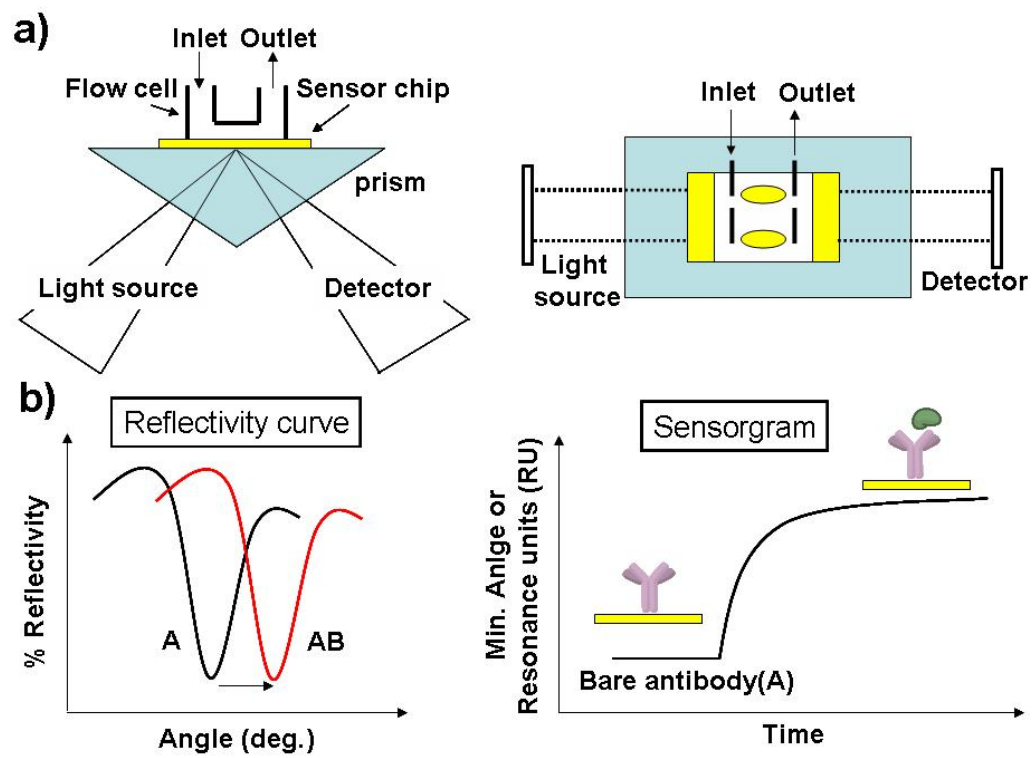


Figure 1.2. SPR sensor in angular configuration with two parallel channels: a) side view and top view, and b) SPR spectroscopy reflectivity curve and sensorgram.

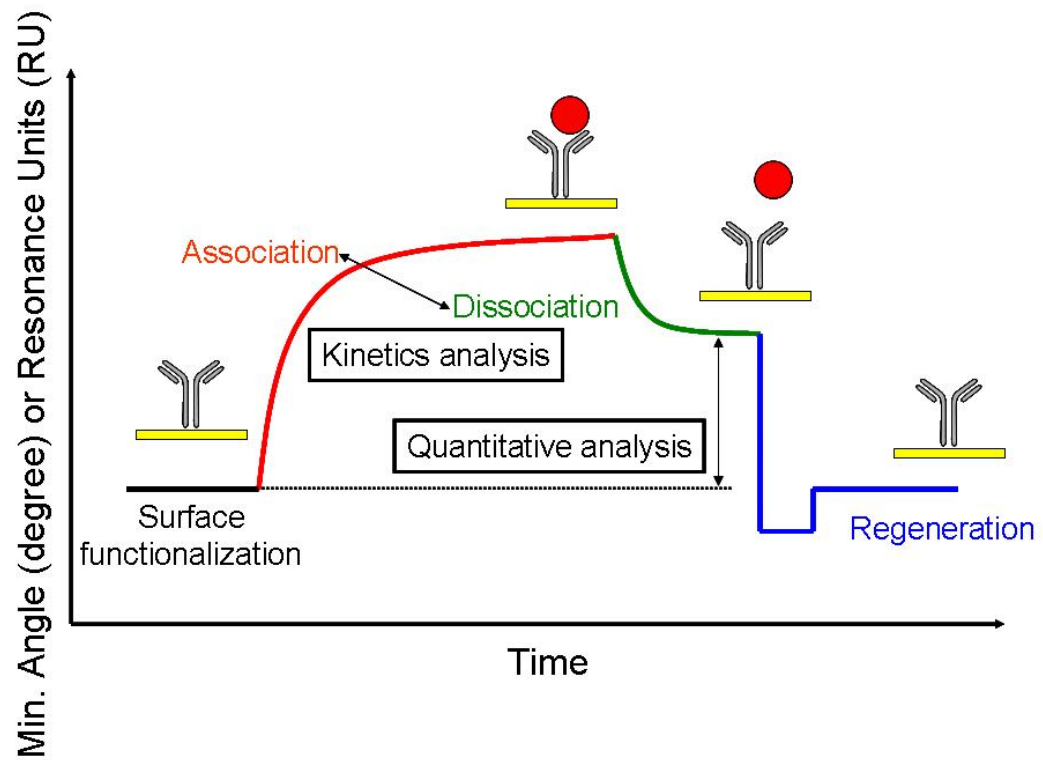


Figure 1.3 The kinetics of an interaction can be determined from the information in a sensorgram.

the spatial capabilities of imaging. SPRi represents a promising and highly versatile affinity sensing platform suitable for an array format. SPRi has been reported for a variety of affinity systems, including DNA/DNA¹⁶, DNA/protein¹⁷, RNA aptamers/protein^{18, 19}, antibody-antigen^{20, 21}, and carbohydrate/protein^{22, 23}. In a typical SPRi instrument (Figure 1.4)²⁴, the reflected light from the illuminated surface is monitored by a CCD detector array. Each pixel on the CCD array corresponds to a specific spot on the gold surface, and provides information about surface mass on that spot. Differences in surface mass across the surface cause differences in refractive index at different lateral locations which result in slight shifts of SPR angles from spot to spot. The binding can be detected in a spatially resolved way by monitoring changes in reflected light intensity at each corresponding pixel on the CCD camera. The CCD camera samples all pixels simultaneously in real time, thus giving SPRi the power for high-throughput studies of multiple binding sites on the arrayed sensor surface.

1.1.4 Surface modification and immobilization techniques

In both SPR and SPRi, receptor molecules have to be immobilized to the metal surface in a way that avoids non-specific adsorption and retains bioactivity of bio-receptors². Developing proper surface chemistry is crucial for a successful SPR/SPRi experiment. Figure 1.5 lists the immobilization strategies to couple biological molecules to surface.

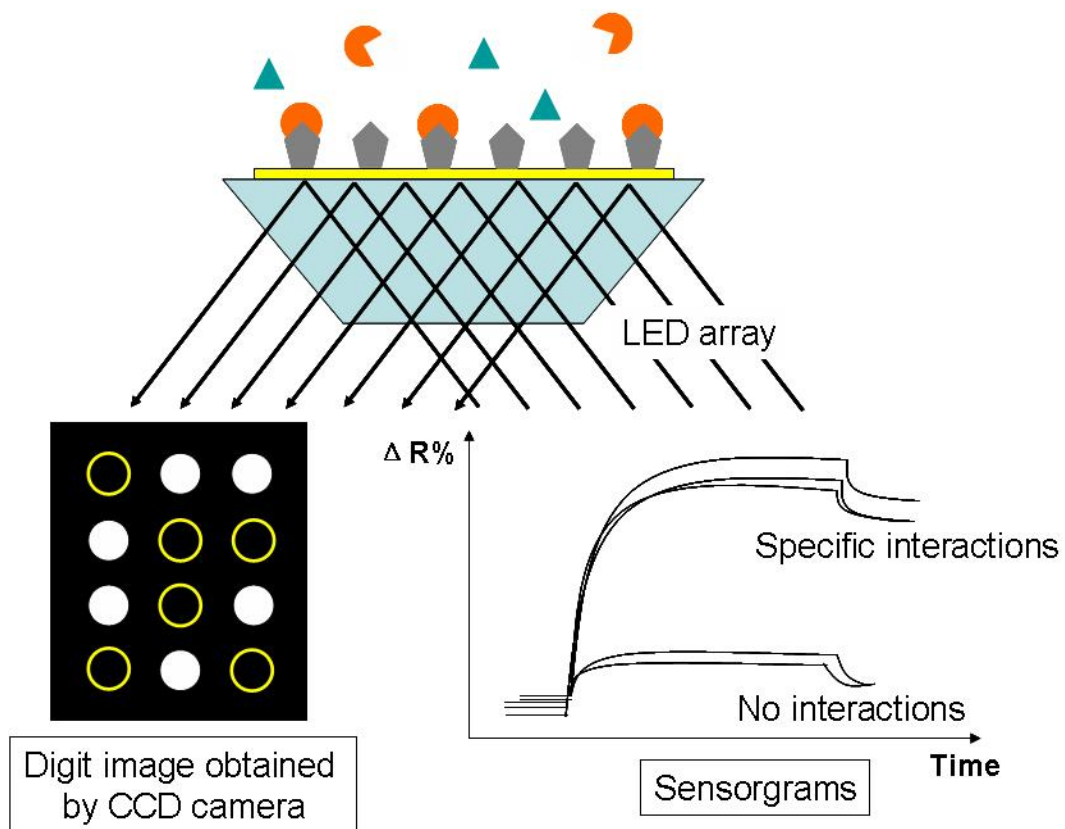


Figure 1.4. A schematic of a standard SPR imaging setup with a differential image and the relative sensorgrams.

Physical adsorption is simple and has the advantage of immobilizing large amount of biomolecules on the surface. Bovin serum albumin (BSA) is attached on gold surface via direct physical adsorption and stays immobilized under mild rinsing conditions. Therefore, antibodies can be immobilized on gold surfaces through antibody-BSA conjugates, the method has been applied in detection of low molecular weight analytes such as TNT²⁵. In addition, avidin has been used as a bridge to connect biotin-antibody to biotin-BSA adsorbed surface²⁶. A problem with this approach is that conjugated antibodies have to be prepared specifically for each analyte, which is technically demanding and time-consuming.

Another favored immobilization method is the use of a self-assembled monolayer (SAM) on the gold surface, which increases the degree of freedom of the probes and consequently, those of binding target molecules. The surface density of receptor molecules can be conveniently controlled with this method. The receptor can be covalently attached via alkanethiols and alkoxylenes containing ω -terminated hydroxylic²⁷, carboxylic²¹ or amine functional²⁸ groups. Carboxylic acid group can be activated by 3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) to form a semi-stable NHS ester, which reacts with an amine in the following reaction to form the amide bond (Figure 1.6a). Thiol modified biomolecules, DNA, or RNA sequences have been directly attached to the gold surface¹⁶ or via connection

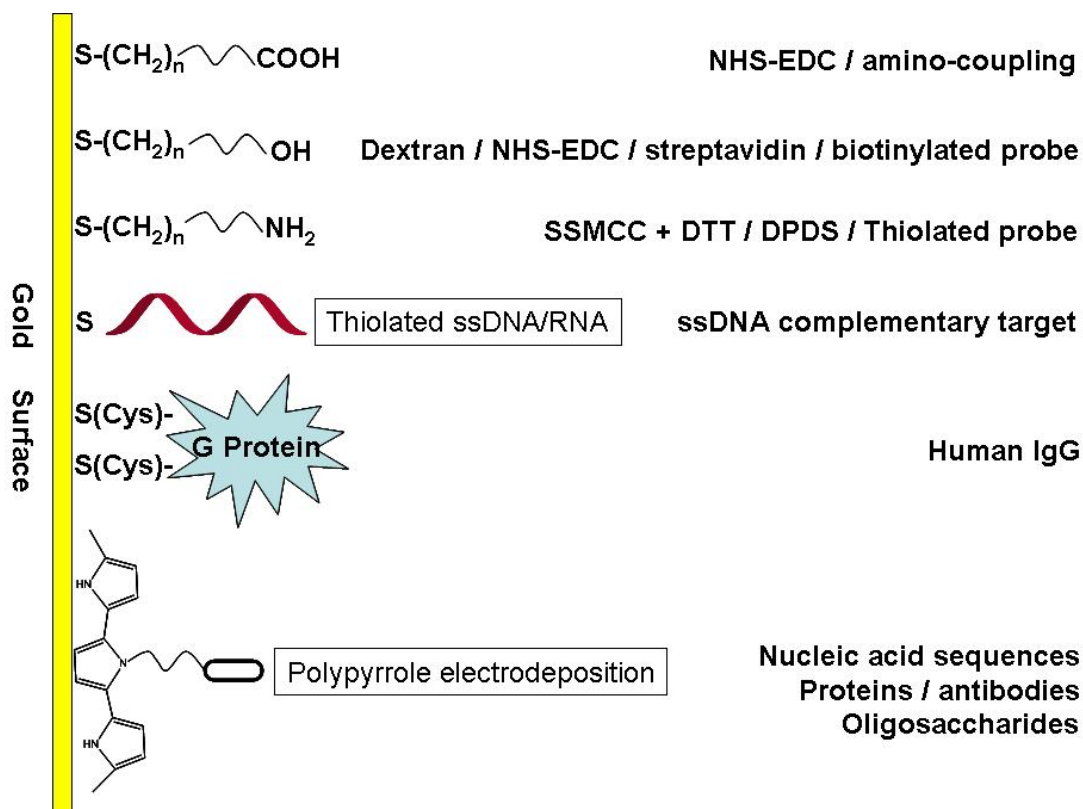


Figure 1.5. Immobilization methods using chemical linkers with self-assembled monolayer and direct attachment of probe to surface using pyrrole-functional biomolecules.

molecules²⁹. An amine terminated alkanethiol (11-mercaptopundecylamine, MUAM) SAM has been reacted with cross linker sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-carboxylate (SSMCC) to form thiol-reactive maleimide-terminated surface. Thiol modified DNA sequences are then spotted and immobilized for detection of complementary DNA sequence (Figure 1.6b). In addition, probe immobilization to polyelectrolyte films such as poly(ethylenimine) (PEI) via electrostatic interactions has also been reported²⁷. Formation of a hydrogel film composed of carboxymethyl dextran covalently linked to the gold surface with the SAM layer is broadly used³⁰.

The principal method to attach protein on surface is via amino-coupling. One limitation of amino-coupling is the lack of control of receptor orientation, since coupling can occur randomly with any amine group on the side-chains²⁴, which may result in a reduction in the biosensor sensitivity and reproducibility. To develop new immobilization procedure to overcome these drawbacks, protein G has been used as an affinity receptor for antibody immobilization, the cysteine residues at N-terminus facilitating orientation of immobilized antibodies^{31, 32}. Another immobilization method is mainly based on polypyrrole. The polymer film consists of pyrrole chains bearing covalently linked oligonucleotides, proteins³³, or oligosaccharides²³. The electropolymerization reaction allows for a very fast coupling of the probes directly to the gold surface, without the need for further chemical linkers.

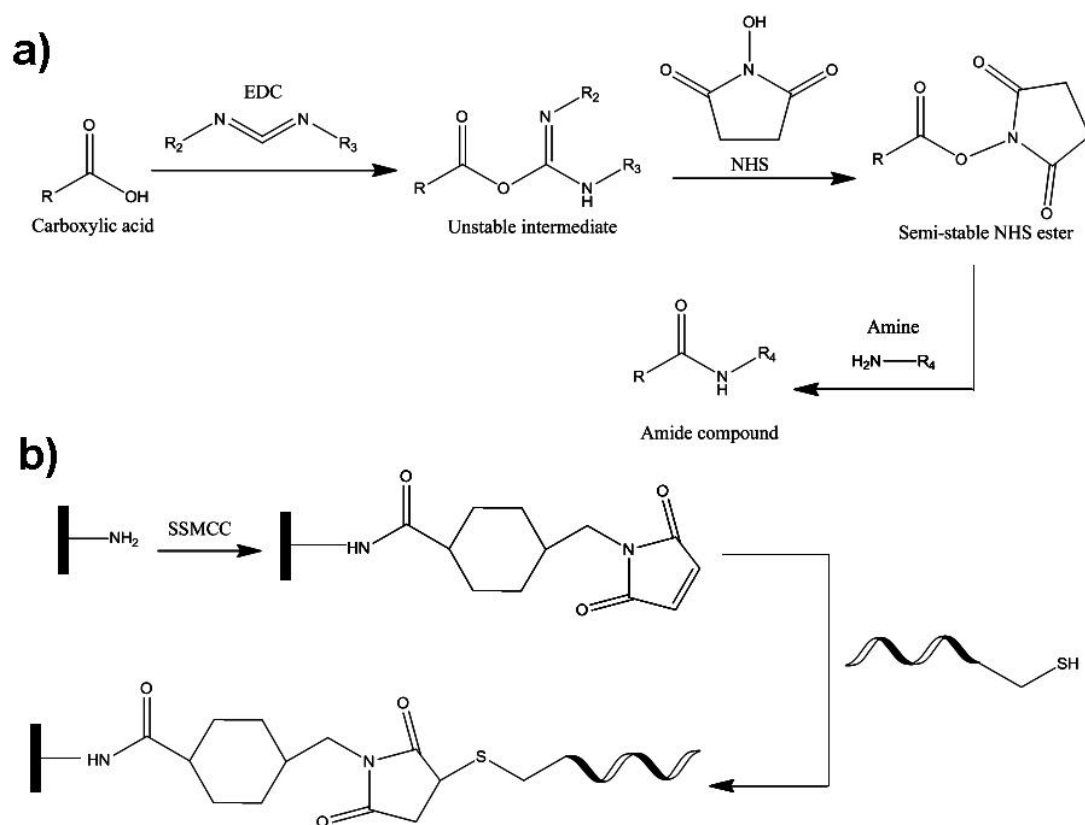


Figure 1.6. Reaction mechanism for a) carboxylic acid activated by EDC/NHS to generate amide compound and b) amine functionalized surface reacted with thiol modified biomolecule with cross linker SSMCC.

1.1.5 Detection sensitivity amplification strategies for SPR and SPR imaging

One of the fundamental issues impeding the further development of microarray-based technologies for biomarker profiling and disease diagnostics is insufficient sensitivity for reliable detection of low-abundant proteins³⁴, especially when operating in complex biological medium with excessive (as much as 10^7) abundant proteins and when the binding affinity to the target is weak (in the μM to mM range). The ability to routinely measure protein targets at femtomolar concentrations and lower is extremely valuable for early disease diagnosis. However, the Kretschmann configuration of SPR presents the physical limitations for performing ultra-sensitive detection.

Several approaches have been reported attempting to increase SPR detection sensitivity with instrumental design and experimental optimization. The measurement position of reflection angle has been optimized to enhance signal-to-noise ratio³⁵. Applying differential processing of two images from two wavelength light sources can significantly improve detection limit of oligonucleotides hybridization³⁶. A paired surface plasma wave biosensor (PSPWB) has been described by generating multiple traversing of optical beam through sensing surface, which not only produces a high detection sensitivity but also provides a large dynamic measurement range³⁷. Grating parameters have been coupled to SPR chips to create a surface plasmon bandgap and detection sensitivity is enhanced when operating near the bandgap³⁸.

Novel surface chemistries, in particular particle attachment, have been extensively investigated for signal enhancement. A layer of protein-Au colloid complexes was used in a measurement that has led to a 25-fold increase in the SPR resonance angle shift³⁹. Layer-by-layer assembly of Au nanoparticles and polyelectrolytes results in a high SPR resonance angle shift under certain solution conditions⁴⁰. With the particle-enhanced sandwich immunoassay, the detection limit of chloramphenicol was enhanced to 0.74 fg/mL⁴¹. In addition, poly(A) tail has been attached to surface immobilized miRNA via polymerase surface reaction, and poly(T)-coated gold nanoparticles have been hybridized to a sensing surface to amplify miRNA binding signals, which attained a 5 attomole detection limit⁴² (Figure 1.7a). In addition, streptavidin-coated latex microbeads have also been employed for the sandwich immunoassay, amplifying the detection limit of prostate-specific antigen (PSA) to 2.4 ng/mL⁴³. Another elegant strategy for SPR signal enhancement is the use of enzymatic amplification. These include measurement of a signal increase induced by localized precipitation^{19, 44} and detection of signal decrease caused by selective hydrolysis of RNA-DNA heteroduplexes^{45, 46}. Hydrolysis enzyme RNase H can recognize DNA-RNA hybrid couple and continuously release surface immobilized RNA, thus DNA detection amplification was achieved via signal decrease from RNA loss⁴⁵. (Figure 1.7b). Recently, polymers have been explored as signal enhancing materials. Aniline monomers have been adsorbed on DNA backbones through

electrostatic interactions for a peroxide-initiated polymerization, improving the detection limit to 0.1 pM⁴⁷. Our lab has developed methods for signal amplification with polymer growth at protein binding sites through atom transfer radical polymerization (ATRP) reaction for detection of cell membrane binding proteins and in an all-aqueous condition^{48, 49}, which allows highly sensitive detection of proteins in a flow-injection based analysis.

1.1.6 Comprehensive bioanalysis: SPR/Mass spectrometry hyphenation

A technique capable of kinetic characterization of biomolecular interactions and identification of interaction partners is of great significance to biomedical research. SPR registers only the total amount of biomaterial retained on the surface. It does not discriminate in the types of molecules it detects. Therefore, events such as multi-protein and nonspecific binding cannot be easily differentiated and can have a detrimental effect on the kinetic analysis⁵⁰. Although specificity in SPR is implied by the immobilized affinity ligand, a second specificity measure/dimension would greatly improve the performance in positive identification. The development of the hyphenation of SPR with mass spectrometry (MS) is set to meet the demand^{51, 52}. Both techniques are well established as stand-alone methods in the field of proteomics and functional genomics. In one hand, SPR allows the kinetic and thermodynamic analysis of biomolecular interactions, with the main advantages of the real-time and label free measurement of

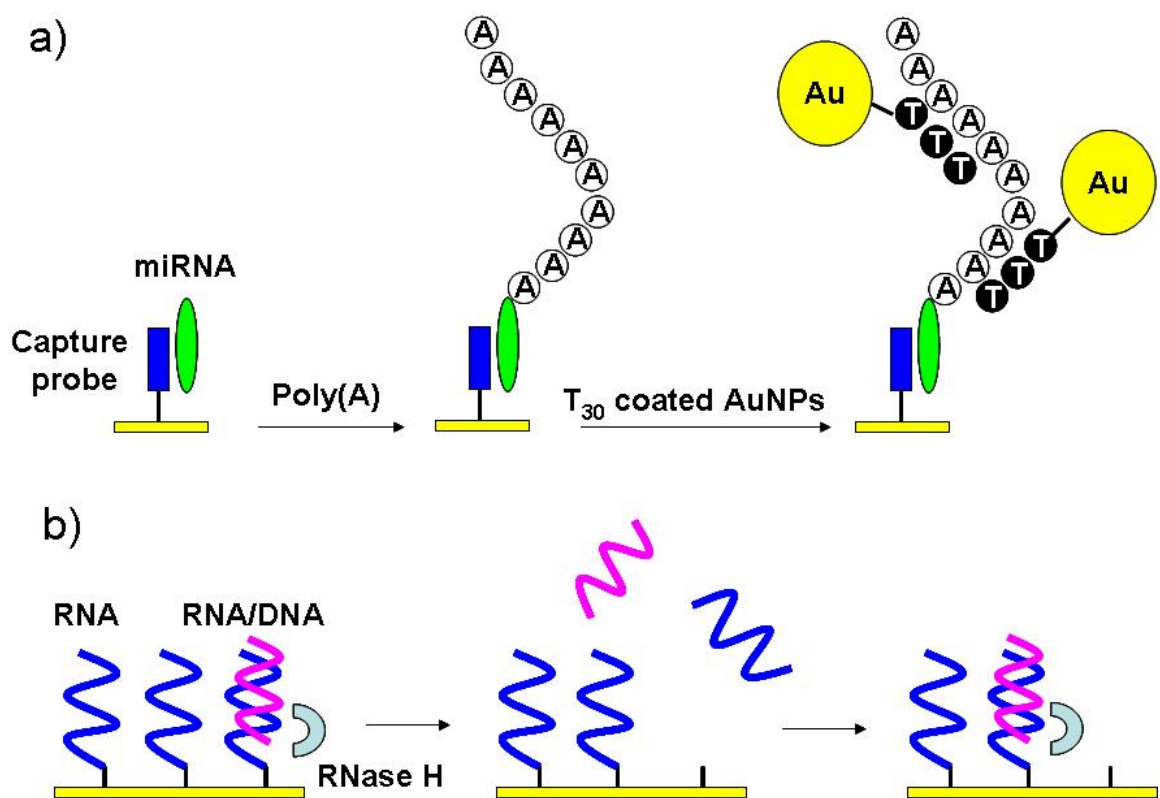


Figure 1.7. SPR signal amplification with a) nanoparticle binding and b) enzymatic reaction.

reaction rate constants (k_{asso} , k_{disso}) from which equilibrium constants (K_a , K_d) can be deduced. Furthermore, SPRi allows high-throughput measurement of interactions *enmasse*, which is significant to large scale analysis. Since SPR detection is nondestructive to the proteins being analyzed, the same proteins that are affinity-retrieved on the SPR chips can be further analyzed via matrix assist laser desorption ionization–time of flight (MALDI-TOF) or electrospray (ESI) mass spectrometry. These MS methods identify the specifically retained ligands through the measurement of their molecular weights and fragmentation patterns^{53, 54}, leading to comprehensive protein analysis.

The SPR-MS technique is still in its early development stage. The interface between SPR and MS can be somewhat complicated, and requires to be well defined. There are two strategies for MS detection of the SPR captured ligands. One is based on the recovery by elution from the biochip surface and their subsequent MS analysis. In this protocol, a capture protein or peptide is immobilized on a sensor chip. Complex biological solution is injected through the chip into the fluidic system. Noninteracting proteins from the biological sample are then washed away, and proteins that have bound to the immobilized ligand are recovered by injection of the elution buffer. These interacting proteins are then recovered and digested into peptides that are separated and analyzed with MS⁵⁵. The eluted ligands obtained by the recovery procedure can be

analyzed using the MALDI⁵⁶⁻⁵⁸, surface-enhanced laser desorption ionization (SELDI)¹⁷, or ESI ion sources⁵⁹. This strategy has been widely employed because most of the SPR-MS coupling devices described in literature operate with a flow cell. However, the recovery procedure is known to be time-consuming and leads to material loss and contamination, while the quantitative elution could be challenging in the case of a high-affinity interaction⁶⁰.

Another strategy involves direct on-chip MS analysis. Given that SPR surface could be easily interfaced with the mass spectrometer, the direct on-chip MS analysis may overcome some limitations mentioned above, and is amenable to high throughput analysis. MALDI appears as the most versatile method for on-chip MS analysis⁶¹⁻⁶³.

The recent introduction of the array format has contributed to a significant improvement in SPR analysis. Unlike the usual flow-cell setup, it allows the study of an important number of interactions in parallel at the same time^{64,65}. The SPR-MS analysis can be operated on biochips in a microarray format, which can be easily inserted into the MS analyzer and allows both SPRi and MALDI-MS measurements directly from the same surface. Short polyoxyethylene chains functionalized surface have been used for protein capture, in situ enzymatic digestion and following tandem MS/MS identification⁶⁰.

1.2 SPR biosensor for lipid membrane interaction

1.2.1 Cell membrane and its components

Cell membranes play key roles in cell life, which defines structure to the cell and controls the transport of ions and molecules between the inside and outside cellular worlds as well as participates in various intra- and extra cellular processes⁶⁶.

The bilayer lipid membrane is only a few nanometers thick, but the assembly is highly complex and dynamic, consisting of two main components: a two-dimensional space made of lipid molecules held together by hydrophobic interactions and self-assembled as a continuous bilayer and proteins embedded within the membrane or transiently associated with it. The major component that forms the bilayer membrane is phospholipids molecules. Phospholipids are a class of lipids consisted of four components: fatty acids, a negatively-charged phosphate group, nitrogen containing alcohol and a backbone⁶⁷.

Figure 1.8 shows the structures of the cell membrane and a typical phospholipids molecule, egg phosphatidylcholine (PC). The two fatty acids form esters with two of the hydroxyl groups of a glycerol. The third hydroxyl group forms another ester bond with the phosphate group. These lipids consist of both hydrophilic (water soluble) and hydrophobic (water insoluble) portion. In the membrane, the phospholipids are stacked with the non-polar hydrocarbon chains pointed to each other while the polar ends pointed

out to form the external surface. The cell membrane may contain other molecules such as carbohydrates and proteins on the surface, which serve as receptor sites for other messenger molecules. Interaction with the cell membrane allows for molecular communication signals to pass from outside to inside of the cell. Lipids in a bilayer are highly mobile in the plane of the bilayer when the temperature is above the characteristic transition temperature, which is in the range of 10 to 40 °C^{68, 69}. When the temperature is below transition temperature, the lipid molecules form a more orderly structure to yield a gel-like solid and lose mobility. The fluidity of biological membranes is a very important characteristic of the biological system because it allows the membrane proteins and receptors to rearrange their conformation and orientation to interact with molecules.

1.2.2 Bilayer vesicles

When lipids are dispersed in aqueous phase, they self-assemble through hydrophobic-hydrophobic interactions spontaneously into a population of vesicles. Under osmotically balanced conditions, the shape of vesicles are spherical thus to maintain the most energy-favorable state⁷⁰. Vesicles can contain a single bilayer referred to as unilamellar or multiple bilayers in a series of concentric spheres structure referred to as multilamellar. The size of these vesicles ranges from tens of nanometers to tens of microns in diameter. Small unilamellar vesicles (SUVs) have size range around 15 to 50 nm, multilamellar vesicles (MLVs) around 100 to 1000 nm, and the size of large

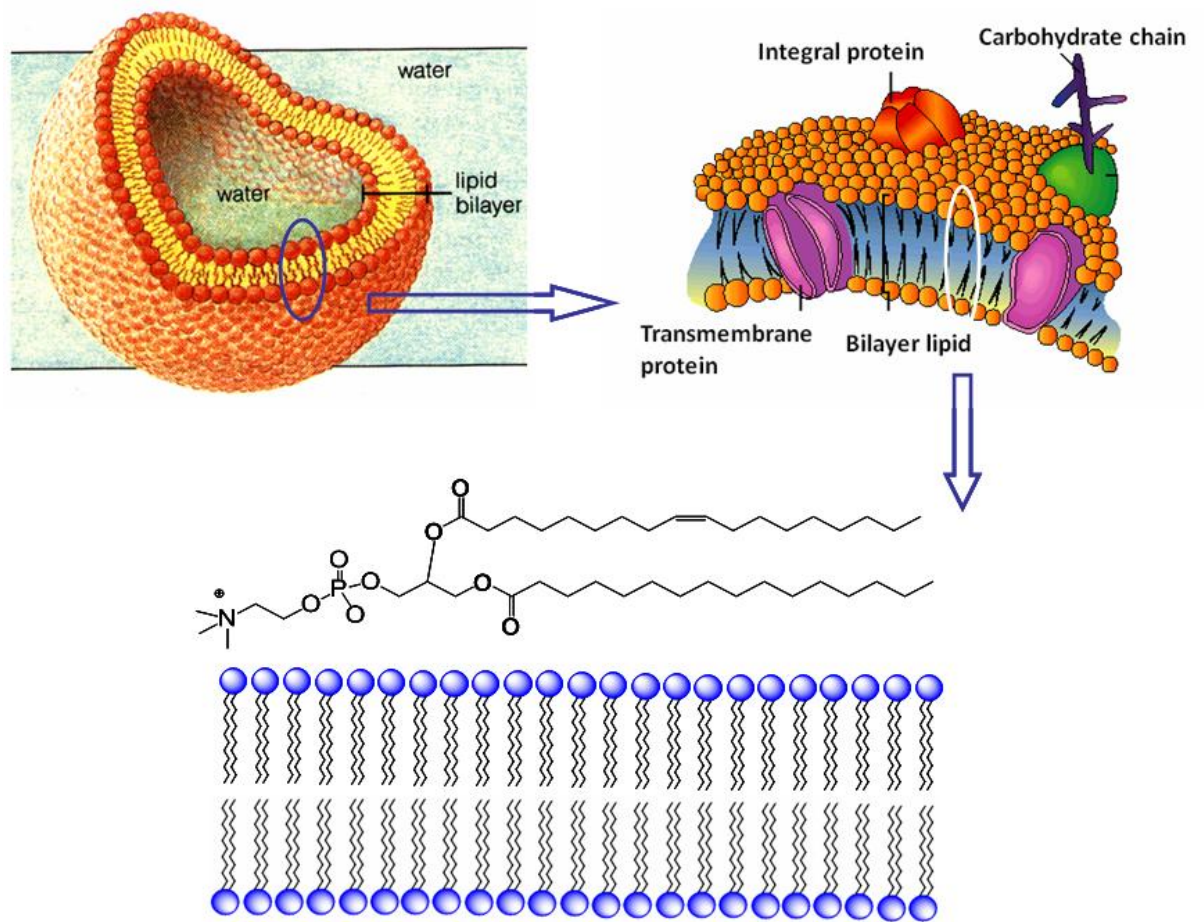


Figure 1.8. Structure of the cell membrane and L- α -Phosphatidylcholine.

unilamellar vesicles (LUVs) is around 1000 nm⁷¹. Vesicles can fuse on hydrophilic surface and form a bilayer structure which is in principal identical to the lipid portion of natural cell membranes, thus it is widely studied as a simplified model of cell membrane. Membrane proteins and receptors can be incorporated into vesicles and this model system can be used to study biological phenomena such as protein-protein, protein-receptor interaction, and ions and molecules transportation through the cell membranes. Vesicles have also been extensively studied as biosensors^{72, 73}, and as vehicle for drug delivery⁷⁴⁻⁷⁶.

Mechanical dispersion is the method which is mostly common used in vesicle preparations. Major techniques of mechanical dispersion include simple shaking, ultrasonication, membrane extrusion and freeze-thaw method⁷¹. Ultrasonication method can be preformed using a strong bath sonicator or a probe sonicator. This method applies high energy to the lipid solution, breaks lipid aggregates into small pieces and usually generates SUVs with sizes below 100 nm. Membrane extrusion is a very gentle method of controlling the size of vesicles. By pushing vesicle solution through a membrane filter of defined pore size, large vesicles are broken into smaller vesicles on the other side of the membrane. After passing through the membrane for multiple times, vesicle populations obtain sizes with an even size limit. Other vesicle preparation methods

include solvent dispersion, which dissolved lipids in an organic solvent first, followed by injected into an aqueous phase⁷⁷, and detergent solubilization, which use detergents to solubilize lipids and form micelles in the solution⁷⁸.

Characterization of vesicles includes entrapment efficiency, lamellarity (number of bilayers in the vesicles) and size of the vesicles⁷¹. Among these, size distribution and average size of the vesicles are of most significance to us. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) are the most frequently used methods to obtain size information. Negative stain TEM is an effective method to obtain the size information of vesicles since vesicles can be visualized under the microscope. The inside of the intact vesicles where is not stained appears bright so the vesicles can be visualized by the contrast with the background⁷⁹. DLS is also a useful method in size determination of the vesicles. When a beam of light passes through a colloidal dispersion, some of the light is scattered by the particles in all directions, the pattern of scattering light depends on the size of the particles^{80, 81}.

1.2.3 Supported bilayer lipid membranes

Surface-confined membrane systems have attracted considerably interest during the past decade. A large number of systems have been described, including solid-supported lipid bilayers⁸²⁻⁸⁵, polymer-cushioned lipid bilayers⁸⁶⁻⁸⁸, hybrid bilayers^{89, 90}, tethered lipid bilayers⁹¹, suspended lipid bilayers^{92, 93}, and supported vesicular layers⁹⁴. Figure 1.9

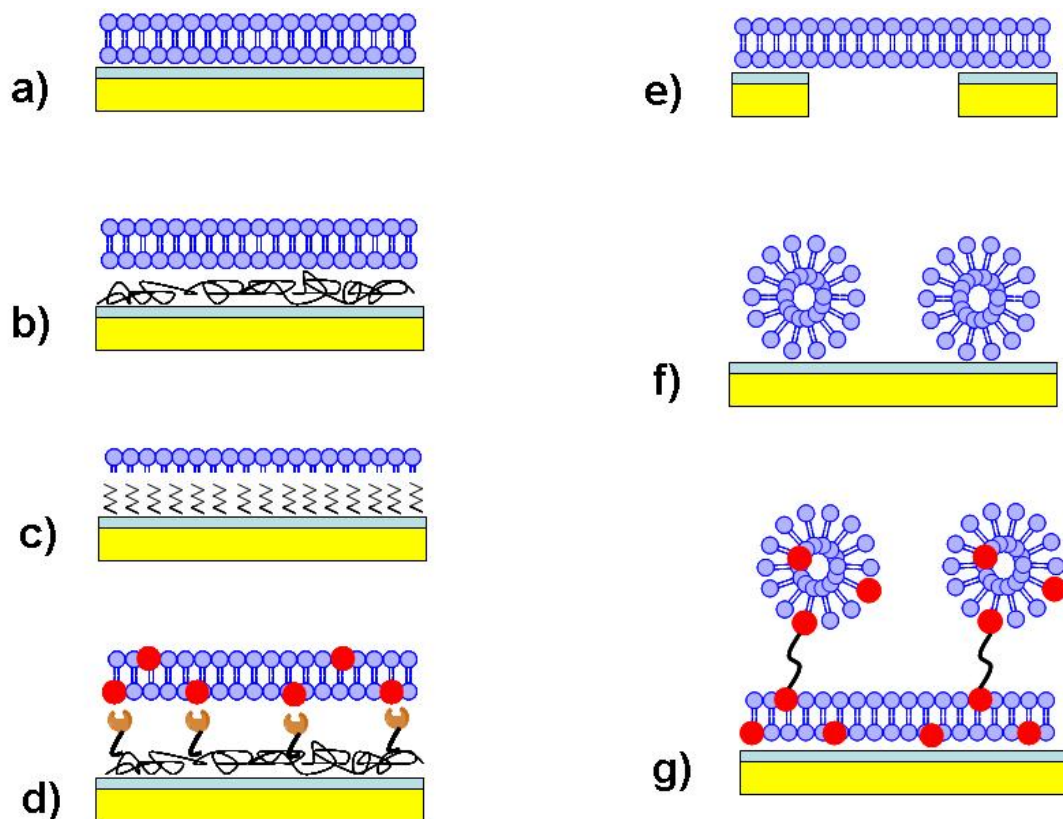


Figure 1.9. Surface-confined membrane models: a) solid-supported lipid bilayer; b) polymer-cushioned lipid bilayer; c) hybrid bilayer, consisting of a self-assembled monolayer (e.g. thiols on Au or silanes on glass or silica) and a lipid monolayer; d) tethered lipid bilayer; e) freely suspended lipid bilayer; f-g) supported vesicular layers.

shows examples of these supported bilayer models⁶⁶. Bilayer lipid systems are widely adopted as cell membrane models to study basic membrane biology and as platforms for biotechnology applications.

Our research interest in cell membrane mimics has focused on solid-supported bilayer membranes. These membrane models are relative robust and stable, and the membrane fluidity is maintained by a 10-20 Å layer of trapped water between the substrate and the bilayer. A schematic diagram of a supported lipid bilayer is shown in Figure 1.10.⁹⁵

There are three general methods for the formation of supported phospholipid bilayers on planar supports for sensor applications. The first method involves the transfer of a lower leaflet of lipids from the air-water interface by the Langmuir-Blodgett technique. This is followed by the transfer of an upper leaflet by the Langmuir-Schaefer procedure, which involves horizontally dipping the substrate to create the second layer⁹⁶. A second method of supported bilayer formation is the adsorption and fusion of vesicles from an aqueous suspension to the substrate surface⁸⁴. A combination of the two methods can be employed by first transferring a monolayer via the Langmuir-Blodgett technique followed by vesicle fusion to form the upper layer⁹⁷. The adsorption and fusion of small unilaminar vesicles is one of the most versatile means for forming solid supported phospholipids bilayers.

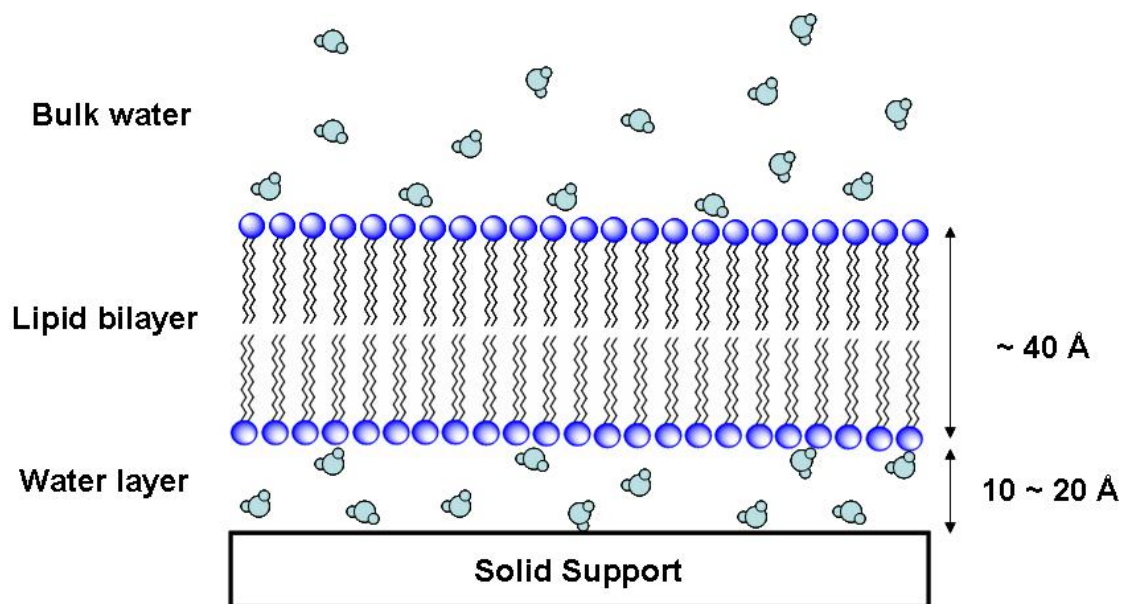


Figure 1.10. Schematic diagram of a solid supported phospholipids bilayer. The membrane is separated from the substrate by a 10-20 Å thick layer of water.

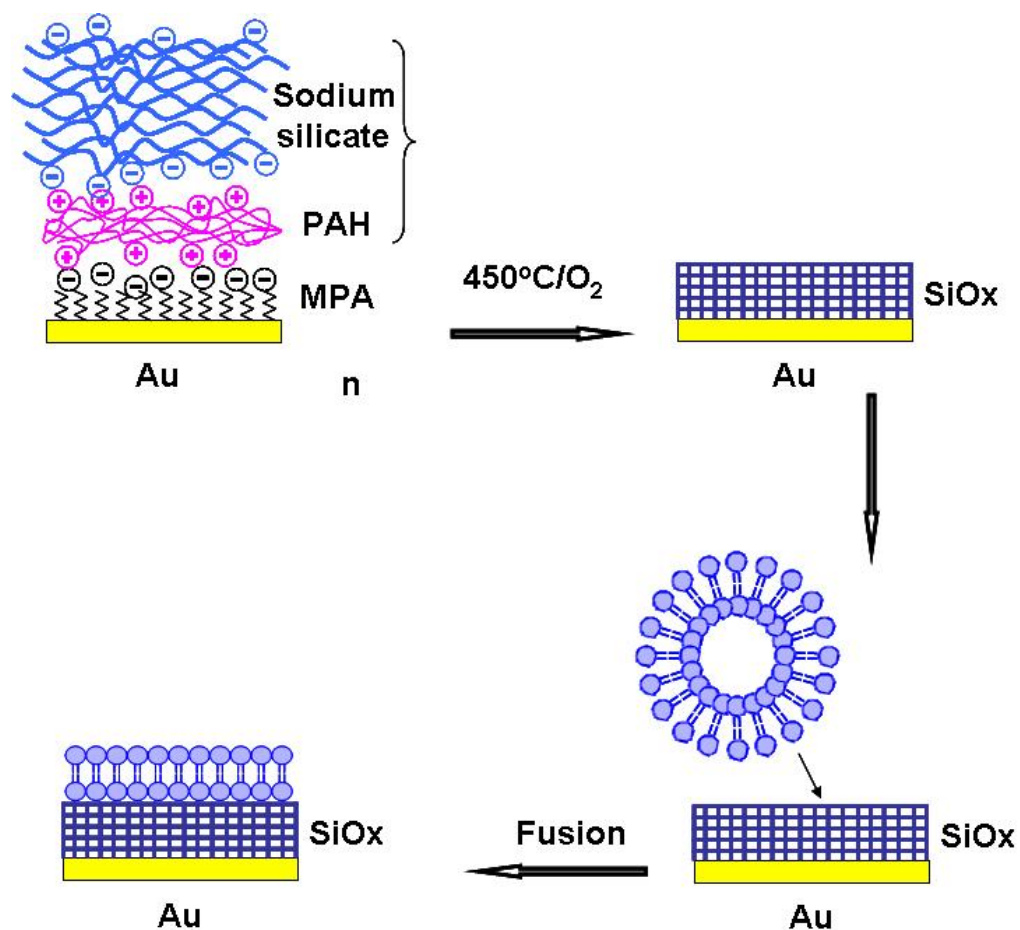


Figure 1.11. Calcinated chip generation with layer by layer assembly of sodium silicate and PAH, and the formation of bilayer lipid membrane on calcinated chip by vesicle fusion.

Using solid support increases the robustness and stability of phospholipids bilayer membrane. In addition, it provides the ability to probe interactions that occur at the membrane surface with powerful analytical techniques that are surface specific (e.g. atomic force microscopy, quartz crystal microbalance, surface plasmon resonance, etc.) QCM and SPR require to be performed on the gold substrate. However, SLBs can not form directly on gold due to its hydrophobic property. The use of self-assembled monolayer is one of the methods to form a hybrid bilayer. Octadecanethiol is a typical choice due to its ability to form tightly packed and well-ordered monolayers, and the fusion of vesicles to octadecanethiol SAMs produces a hybrid bilayer membrane⁹⁸. The physical properties of a hybrid bilayer can be altered through the use of different alkanethiols, lipids, and membrane additives such as sterols and proteins. Increasing the chain length of the alkanethiol or phospholipids results in a thicker membrane, thus decreasing its capacitance⁹⁹. Altering the composition of the vesicles can also change the properties of hybrid membranes. Incorporation of ligand-conjugated lipids into the membranes is useful for investigation of binding kinetics and multivalent interactions. Poly(dimethylsiloxane) (PDMS) coating is typically hydrophobic. Oxygen plasma treatment makes surface hydrophobic and thus suitable for bilayer formation. Study has shown once a bilayer has formed, the surface stays hydrophilic as long as the bilayer keeps hydrated^{100, 101}. Our group has demonstrated that a thin (5-50 nm) glassy layers can

be produced on gold substrates using layer-by-layer assembly of sodium silicate and poly(allylamine hydrochloride) (PAH) followed by calcinations. The nanoscale silicate coatings are stable in buffer, allowing vesicle fusion to bilayer lipid membrane and SPR measurement^{102, 103} (Figure 1.11).

The 10-20 Å water layer that resides between a phospholipids bilayer and a solid support provides lubrication and maintains sufficient mobility for the lipid molecules¹⁰⁴. However, to further improve lateral fluidity and the insertion of membrane biomolecules, polyion “cushions”¹⁰⁵ have been used, which provide a hydrophilic “aqueous” layer between the surface and the lipid membranes. The addition of a polymer layer effectively decouples the membrane from the surface and provides the appropriate environment for transmembrane proteins. It is important that the polymer cushion have the ability to swell in an aqueous environment and have minimal disruptive interaction with the bilayer and any other reconstituted membrane components¹⁰⁶. It has been observed that the quality of the supported membrane can also be affected by the degree of swelling of the polymer layer prior to bilayer deposition¹⁰⁷. Another potential advantage of polymeric supports is the ability to selectively capture biomolecules from solution. Thermoresponsive polymers have been extensively explored for this purpose^{108, 109}.

1.2.4 Artificial receptors and molecular recognition

The surfaces of mammalian cells are decorated with a wide variety of membrane-bound receptor molecules. These receptors act as sensors for and transporters of small molecules in the extracellular environment¹¹⁰. The nature receptors have inspired the discovery of new methods to transport polar drug candidates across hydrophobic membrane bilayers, a process of great importance to medicinal chemistry^{111, 112}. Natural receptors such as glycolipids¹¹³ or polypeptides¹¹⁴ are responsible for recognition of the target and target drug delivery by covalently linking the drug target to a suitable vector^{115, 116}.

Artificial receptors have been designed and studied to mimic the functions of natural receptors¹¹⁷. These receptors can be covalently attached to a lipid or steroid derivative, which is incorporated in a synthetic membrane where the recognition motif is displayed above the membrane surface to bind to the target¹¹⁸. An alternate method is the incorporation of a defined binding pocket inside the membrane bilayer. This strategy is employed by membrane-penetrating proteins¹¹⁹ and transmembrane pore-forming peptides¹²⁰; the host is incorporated in the membrane itself and displays a cavity that allows polar substrates to be shielded from the lipophilic membrane¹²¹ (Figure 1.12a). This type of binding motif shows a greater range of application than receptors created via the covalent derivatization of steroids and lipids.

Cavitands are artificial receptors well-known as protein mimics. They provide a cavity that can selectively recognize molecules of the correct shape if they contain a thin layer of positive charge at their surface. Synthetic cavitands have been incorporated into a membrane bilayer and recognize their desired targets¹²² (Figure 1.12b). Excellent selectivity for substituted trimethylammonium salts is possible in both water^{123, 124} and organic solvents¹²⁵. Recognition of hydrocarbons¹²⁶ and steroids¹²⁷ via the hydrophobic effect has also been reported. Our lab has studied the molecular recognition in a membrane-mimicking setting via incorporation of cavitand into a membrane bilayer¹²⁸.

1.3 Atom transfer radical polymerization (ATRP) reaction

As discussed in 1.1.5, several amplification strategies have been employed to enhance SPR signal for ultrasensitive protein detection, which includes nanomaterial binding, enzymatic reaction and polymer deposition. Direct triggering of *in-situ* polymer growth from protein binding site is easy to operate and can provide high specificity, thus is suitable for signal amplification in complex biological systems. An ATRP method has been developed for *in-situ* polymer growth that can be applied for ultrasensitive protein detection in proteomics and monitoring molecule interaction in a cell membrane mimic environment.

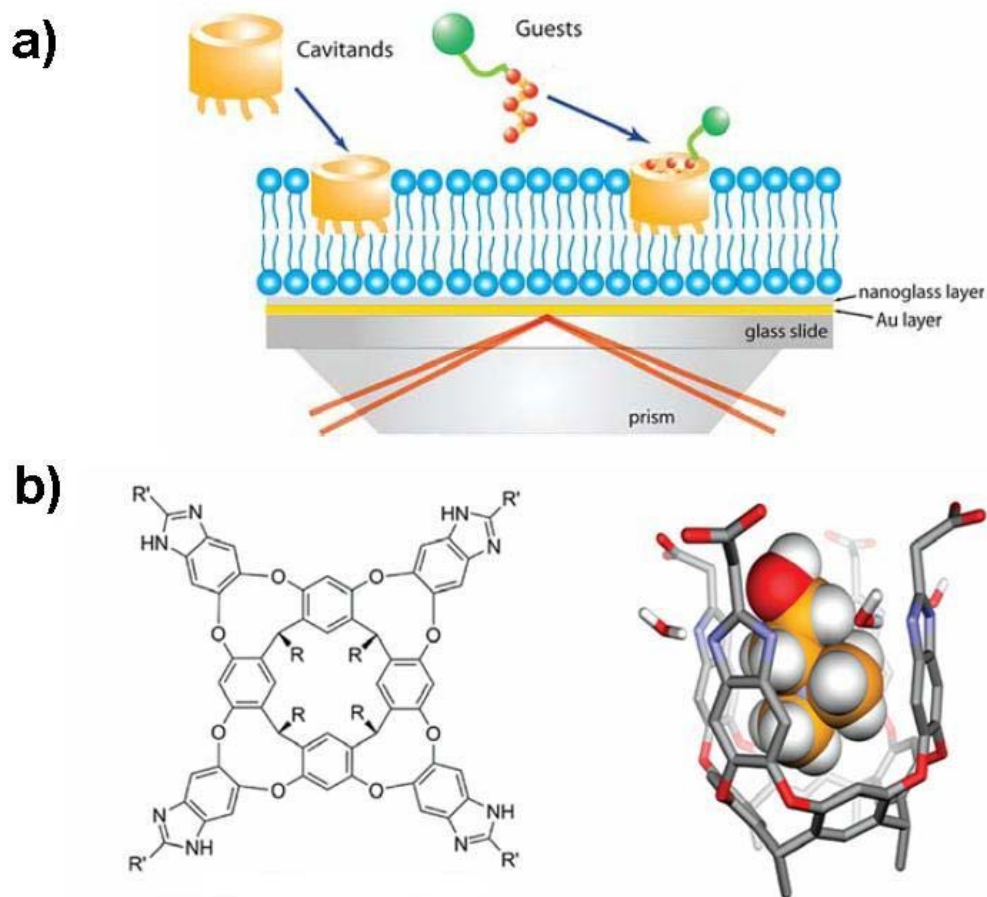


Figure 1.12. a) A Cartoon representation of guest binding to cavitand incorporated in a PC bilayer membrane. b) structure of cavitand and the conformation of cavitand with the bound guest trimethylammonium salt.

1.3.1 ATRP reaction principle

Living polymerizations are chain-growth processes that proceed in the absence of irreversible chain transfer and chain termination, which allow well-defined polymers to be prepared. If initiation is complete and exchange between species of various reactivities is fast, one can adjust the final average molecular weight of the polymer by varying the initial monomer-to-initiator ratio while maintaining a narrow molecular weight distribution, as well as control over the chemistry and structure of the initiator and active end group¹²⁹. A new class of controlled living radical polymerization has been developed, known as atom transfer radical polymerization (ATRP)^{130, 131}.

Control of molecular weight in ATRP is possible due to the fast activation/deactivation cycles (Figure 1.13) that ensure a low stationary concentration of radicals thereby minimizing termination reactions¹³². In ATRP, the initiator/growing chain with the halide end group is activated by a Cu(I) species (activator) giving rise to the growing chain radical, $P_n^\bullet$, which is rapidly deactivated by Cu(II)X (deactivator), and this dormant species can then be activated reversibly to yield free radicals again. In this manner, a small concentration of radicals can be employed to propagate a large number of chains. For an appropriate degree of control, a sufficient concentration of deactivator species must be present in the polymerization medium. There are two necessary conditions for an effective equilibrium. First, the equilibrium between dormant and active (free-radical)

species must lie strongly to the side of the dormant species to assure that the overall concentration of radicals will remain very low and that the rate of irreversible termination will be negligible relative to the apparent rate of polymerization. Second, the rate of exchange between dormant and active species must be faster than the rate of propagation to assure that all polymer chains have an equal probability of adding monomer¹²⁹.

An ATRP system consists of an initiator, a metal halide complex with some ligands, and a monomer. The main role of the alkyl halide (RX) species is to determine the number of initiated chains. The halide group, X, must rapidly and selectively migrate between the growing chain and the transition-metal complex, bromine and chlorine are the halogens that afford the best molecular weight control¹³³. The structure of the alkyl group, R, in the initiator should be similar to the structure of the dormant polymer species. The catalyst must show selectivity for atom transfer and therefore possess a low affinity for alkyl radicals and the hydrogen atoms on alkyl groups. The majority of work has been performed using the copper-based catalyst. ATRP can be used to polymerize and copolymerize a wide variety of monomers, including styrene¹³⁴, methyl methacrylate¹³⁵, methyl acrylate¹³³, acrylonitrile¹³⁶ and their derivatives with accurate control over the molecular weight and molecular weight distribution of the final polymer. The reaction conditions are not very stringent; many types of functional groups and polymerization

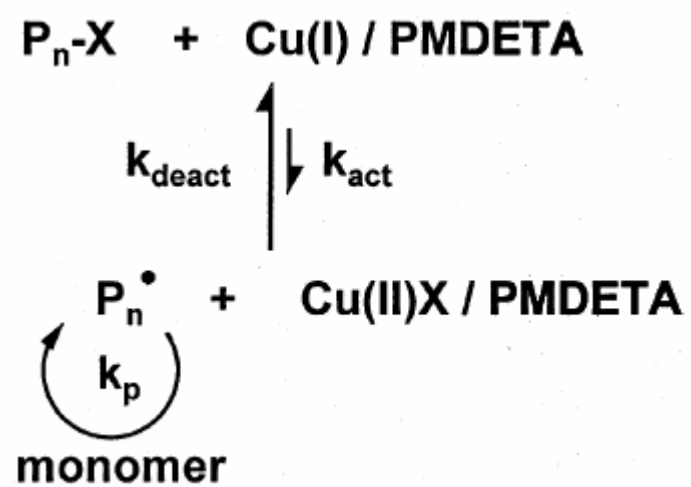


Figure1.13. Schematic of the reaction mechanism of ATRP.

additives can be tolerated. The composition, functionality, and architecture of the final polymer can be controlled through variations in the side groups, end groups, and initiator structures.

1.3.2 Surface initiated polymer growth

ATRP reactions have been used to functionalize surfaces. Polymers grafted densely onto solid substrates have attracted broad attention as a novel method to modify surfaces owing to its potential applications in lithography, corrosion resistance, increased bio-compatibility of materials, and fabrication of electronic devices¹³². An overview of techniques for producing grafted polymer layers is shown in Figure 1.14¹³⁷. Through the immobilization of end-functionalized polymer chains (grafting-to approach), grafted polymer takes mushroom configuration and has limited packing density on surface. High grafting densities can be readily obtained with surface initiated polymerization, in which grafted chains experience strong lateral steric repulsion and demonstrate stretched state, thus aids the production of relatively thick films. Increasing the grafting density can significantly improve film properties such as protein resistance¹³⁸ and lubricity¹³⁹. The covalently immobilized small-molecule initiators can be replaced by macro-initiators with weaker non-covalent binding to the surface such as hydrophobic, hydrogen bonding or electrostatic interactions, which provides high-molecular-weight polymer chains.

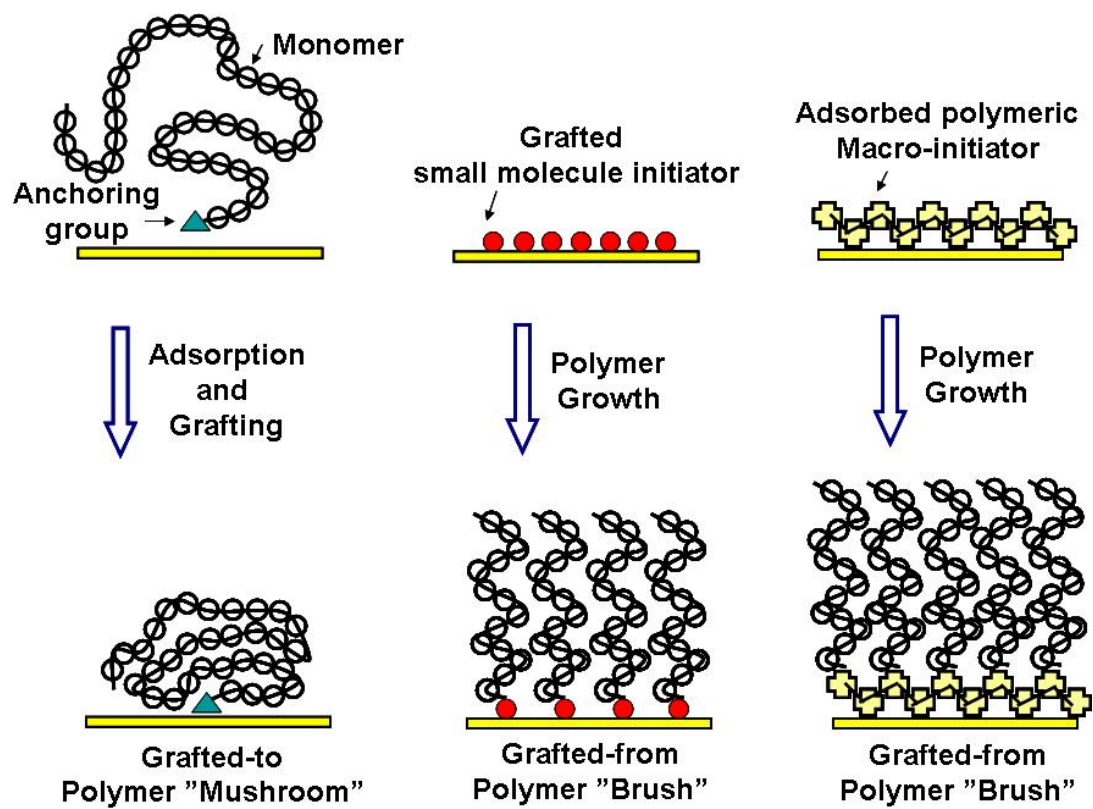


Figure 1.14. Overview of techniques for producing grafted polymer layers.

The majority of reported surface initiator polymerizations require surface functionalization using small-molecule initiators. Anchoring chemistries usually fall into two broad classes: thiol-gold (or other noble metal) bonding¹⁴⁰ or silane-silane bonding^{141, 142}. In addition, several alternative routes have been proposed for initiator syntheses¹⁴³⁻¹⁴⁵. Instead of directly immobilizing synthesized initiator to the substrate, the functional groups of self-assembly monolayer can be converted to initiator by coupling with other molecules. 2-bromoisobutyryl bromide (BIBB) is broadly used to obtain an amide alkoxysilane initiator for atom transfer radical polymerization (ATRP) in one step¹³⁷. By reacting with BIBB, surface bound amine groups¹⁴³ or hydroxyl groups¹⁴⁴ can be converted to initiators and triggered ATRP reactions. “Click chemistry” has also been coupled with ATRP to graft side chain poly(2-hydroxyethyl methacrylate) (PHEMA) to PEG networks¹⁴⁵.

1.3.3 Application of ATRP reaction in biological system

A number of ATRP reactions are known for effective polymerization in aqueous solution and at room temperature¹⁴⁶, and have high tolerance of surrounding functional groups. This property renders the reaction to be used along with biomolecularly functionalized surfaces. Brushes based on poly(ethylene glycol) methacrylate, poly(ethylene glycol) methyl methacrylate, and poly(sulfobetaine) methacrylate have been used as biomimetic fouling-resistant materials to suppress nonspecific protein

adsorption¹⁴⁷. With the specific binding of synthesized initiator to target analyte, localized ATRP polymer growth has been exploited as amplification tools for high sensitive colorimetric DNA detection¹⁴⁸ and SPR protein detection⁴⁸. Brushes generated from thermosensitive polymers such as N-isopropylacrylamide have also been used for the programmed adsorption and release of proteins^{149, 150}.

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Chapter 2 Highly Sensitive Detection of Protein Toxins by Surface Plasmon Resonance with Biotinylation-Based Inline ATRP Amplification

2.1 Introduction

Surface plasmon resonance (SPR) has been widely used as a powerful analytical technique for study of a broad range of biomolecular interactions¹⁻⁶. Label-free and capable of real-time detection, SPR allows for measurement of analyte concentration and binding kinetics⁷ in a fast, convenient, and nondestructive fashion. Recently, spectroscopic SPR and a closely related technique, imaging SPR, have been further adapted as affinity detection techniques in proteomic and genomics field, especially in protein conformation study⁸, biomarker profiling, aptamer selections⁹ and antibody selections¹⁰, which have produced high affinity ligands that specifically recognize protein targets. Using the nondestructive nature of the method, captured proteins and tissue extracts¹¹ on a SPR sensing surface have been subjected to mass spectrometric analysis with and without elution of bound material, showing potential of developing hyphenated techniques with SPR.¹² In both spectroscopic and imaging SPR, the detection of analytes and analyte interactions on a functionalized surface is achieved through monitoring, via the aid of optical couplers, of the changes of the film thickness and refractive index due to biomolecular binding. The Kretschmann configuration of SPR, which is the most widely employed setup, utilizes a thin (approximately 50 nm) layer of a noble metal (usually gold) deposited on a glass substrate that is attached to a prism¹³. This arrangement offers the convenience of generating surface plasmons with simple optical

components but also presents the physical limitations for performing ultrasensitive detection. As a result, one of the fundamental issues impeding the further development of SPR application in proteomics is the lack of sufficient sensitivity to reliably detect low-abundant, trace amount of proteins, especially those in complex biological solutions where there is a huge excess of interference proteins present. The ability to carry out highly sensitive routine protein measurements for the SPR techniques at picomolar concentrations and lower would be extremely valuable¹⁴.

Several approaches have been reported attempting to increase SPR detection sensitivity with instrumental design and experimental optimization, which include signal-to-noise optimization¹⁵; use of two wavelength light sources for differential images¹⁶; multiple traversing of optical beam through sensing surface¹⁷; and use of grating coupled SPR chips that limit propagation of surface plasmons¹⁸. However, these methods often suffer the drawback of limited linear range, and the enhancement is only incremental, which is not sufficient enough for ultra sensitive detection. Novel surface chemistries, in particular particle attachment, have been extensively investigated for signal enhancement. For instance, a layer of protein-Au colloid complexes was used in a measurement that has led to 25 fold increase in the SPR resonance angle shift¹⁹, and a layer-by-layer assembly of Au nanoparticles and polyelectrolytes results in high SPR resonance angle shift under certain solution conditions²⁰. With the particle-enhanced sandwich immunoassay, the solution protein concentration of human immunoglobulin G in the picomolar range could be detected¹⁹, and the detection limit of chloramphenicol was enhanced to 0.74 fg/mL²¹. In addition, poly(T)-coated gold nanoparticles have been hybridized to a sensing surface

to amplify signals from surface-bound miRNA, which attained a 5 attomol detection limit²². Streptavidin-coated latex microbeads have also been employed for sandwich immunoassay, amplifying the detection limit of prostate-specific antigen (PSA) to 2.4 ng/mL²³. Another elegant strategy for SPR signal enhancement is the use of enzymatic amplification²⁴⁻²⁷. These include measurement of signal increase induced by localized precipitation^{24, 25} and detection of signal decrease caused by selective hydrolysis of RNA-DNA heteroduplexes^{26,27}. In addition, polymers have been explored as signal enhancing materials as well. Aniline monomers have been adsorbed on DNA backbones through electrostatic interactions for a peroxide-initiated polymerization, improving the detection limit to 0.1 pM²⁸. Despite these efforts, generic methods allowing signal enhancement for highly sensitive detection of proteins with a broad, versatile fashion in flow-injection based analysis are still lacking.

In this work, we report the development of a polymer-based amplification strategy for a generic and highly sensitive SPR detection of protein molecules. The method is based on the atom transfer radical polymerization (ATRP) reaction, a controlled radical-based process with repetitive addition of monomers to radicals generated from dormant alkyl halides in a reversible redox process²⁹⁻³¹. ATRP has been used to graft polymer brushes on a solid support and yield polymers with high molecular weight, low polydispersity, and controllable thickness and density³². Brushes based on poly (ethylene glycol) methacrylate, poly (ethylene glycol) methyl methacrylate, and poly sulfobetaine methacrylate have been used as biomimetic fouling-resistant materials to suppress nonspecific protein adsorption³³⁻³⁵. Brushes generated from thermosensitive polymers

such as N-isopropylacrylamide have been used for the programmed adsorption and release of proteins^{36,37}. A number of ATRP reactions are known for effective polymerization in aqueous solution and at room temperature³⁸, and has high tolerance of surrounding functional groups. This property renders the reaction to be used along with biomolecularly functionalized surfaces, and the localized polymer growth has been exploited as an amplification tool for colorimetric DNA detection^{39,40}. Scheme 1 shows the general strategy used in this work for surface initiated ATRP amplification of protein detection with SPR. To specifically localize the initiator onto the protein site where signal enhancement is desired, a biotinylated initiator for poly(hydroxyl-ethyl methacrylate) (PHEMA) is synthesized and used along with neutravidin for its attachment to the target proteins via the biotinylated antibody (biot-IgG). The wide availability of biot-IgG against various biomolecules would make this method broadly applicable to almost any proteins of interest. In this work, bacterial cholera toxin (CT) is chosen as the model system because of its biological significance and availability of pure samples and antibody. Pertinent experimental conditions such as deoxygenation steps to suppress oxygen interference for flow-injection analysis (FIA) and use of PEGamine reagent to passivate sensing interface to eliminate nonspecific interaction were developed. In addition, optical and AFM microscopy are used to characterize the polymeric thin films formed on the protein sites through ATRP steps.

2.2 Experimental

2.2.1 Materials

(+)-Biotin was purchased from Fisher Scientific. NeutrAvidin was obtained from Thermo Scientific (Rockford, IL). Cholera toxin (CT), 11-mercaptoundecanoic acid (MUA), 1,1'-carbonyldiimidazole, 2-(2'-aminoethoxy)ethanol, 2-bromo-2-methyl propionic acid (BIBB), 1,3-dicyclohexylcarbodiimide, 4-(N,N-dimethylamino)pyridine, 2-bromoisobutyryl bromide, 2-hydroxyethyl methacrylate (HEMA), CuBr, 2,2'-bipyridyl (bpy), L-ascorbic acid (AA), and triethylamine (TEA) were purchased from Sigma-Aldrich (St. Louis, MO). Biotinylated rabbit anti-cholera serum was from ViroStat (Portland, Maine). O-(2-Aminoethyl)-methylpolyethylene glycol (PEGamine) 750 was obtained from Fluka. N-hydroxysuccinimide and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride were purchased from Acros. All proteins solutions were prepared in 20 mM phosphate buffered saline (containing 150 mM NaCl, pH 7.4).

2.2.2 Synthesis of biotinylated ATRP initiator

The biotinylated ATRP initiator was synthesized according to a published procedure⁴¹. Briefly, biotin was activated by 1,1'-carbonyldiimidazole, followed by its coupling to a hydrophilic linker of 2-(2'-aminoethoxy)-ethanol to form biotinylated alcohol. Esterification of biotinylated alcohol with 2-bromo-2-methyl propionic acid was carried out in the presence of 4-(N,N-dimethylamino)pyridine (DMAP) and 1,3-dicyclohexylcarbodiimide (DCC), which afforded the biotinylated ATRP initiator in a 43% yield after purification by flash chromatography. The compound was verified by Hi Res MS and NMR.

2.2.3 AFM and optical microscopy

Atomic force microscopy (AFM) images were obtained using a Veeco Dimension 5000 atomic force microscope (Santa Barbara, CA) with manufacturer-provided software. All images were obtained in tapping mode. Optical microscopy images were obtained using Zeiss AXIO imager optical microscope under dark field condition.

2.2.4 SPR analysis of protein toxins and signal amplification with ATRP

A dual channel SPR spectrometer NanoSPR-321 (NanoSPR, Addison, IL) with a GaAs semiconductor laser light source ($\lambda=670$ nm) was used for all SPR measurements. The device comes with a high-refractive index prism ($n=1.61$) and 30 μ L flow cell. SPR gold chips were fabricated with a 2-nm thick chromium adhesion layer, followed by deposition of a 46-nm thick gold layer via e-beam evaporation onto cleaned BK-7 glass slides.

Surface interaction and modification were monitored using the angular scanning mode around the minimum angle. After immersed in a piranha solution (7:3 v/v, $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$) (Caution!) for 1 min to remove inorganic and organic contaminants on the surface, the gold substrate was incubated in 1 mM MUA ethanol solution for 18 hours to form a self-assembled monolayer with carboxyl functional group on the surface. After extensive rinsing with copious ethanol and DI water, the chip was dried under a N_2 stream. The gold substrate was then clamped to a flow cell on a prism. To activate the carboxyl acid group, EDC (0.4M)/NHS (0.1M) solution was injected into the flow cell and incubated for 30 min. After rinsing with water, varied concentrations of CT in PBS was injected and incubated for 30 min to allow formation of covalent amide linkages. Passivation of

the unused activated carboxyl groups was performed by incubation with 4 mM PEGamine solution for 20 min, which was followed by a 10-min rinsing to eliminate any residual CT in solution. The binding assay was carried out with injection of 0.1 mg/mL biotin-anti CT and 15 min incubation. Following this, 0.25 mg/mL neutravidin was injected and incubated for 10 min. Finally 0.5 mg/mL freshly prepared biotin-initiator was injected, incubated for 20 min before running a 10 min buffer rinsing.

The first stage amplification with ATRP was conducted with an aqueous solution of 20% HEMA monomer and catalyst (9mM CuBr/18mM 2,2'-dipyridyl) that was purged with nitrogen for 30 min to reduce the amount of O₂ present. After incubation for 30 minutes, the polymerization was stopped by removal of the HEMA/catalyst mixture with PBS buffer. In the second-stage ATRP reaction, subsequent anchoring of additional initiators on PHEMA formed during the first-stage ATRP was used. Direct coupling of 2-bromoisobutyryl bromide to the hydroxyl groups on the PHEMA side chains was achieved by using a solution of 0.08 M 2-bromoisobutyryl bromide in 0.1 M TEA, similar to a previously reported procedure³⁹.

2.3 Results and Discussion

2.3.1 Biotinylated initiator for protein detection and in-line ATRP process

Previous work has used bromoisobutyryl NHS ester to link the initiator to DNA for site-specific ATRP amplification that relied on DNA hybridization⁴² and for peptides on a solid support⁴³. To develop a direct and flexible method for detection of protein samples, the ATRP initiator has to be fixed to the specific protein site through a broadly available attachment scheme. In this work we developed the attaching scheme to localize

the initiators through the well-defined biotin-avidin interaction (Figure 2.1). Two consecutive stages are thus involved in the method: specific initiator binding via biotinylated antibody and local polymer growth. For simplicity of the experiment, CT is covalently linked to surface to demonstrate the feasibility of polymer-based signal amplification in SPR. Essentially all capture schemes including sandwiched assays and competition assays could be adapted for enhanced detection. To eliminate nonspecific adsorption the free, activated carboxyl acid groups of MUA were blocked by PEGamine. The surface attached CT was recognized by injection of biotinylated anti-CT. Then neutravidin was added that specifically binds to the exposed biotin tag. A high concentration of neutravidin was used to ensure effective binding and the captured neutravidins have additional binding site available for biotin-initiators in the following step. The initial catalyst solution was prepared using literature protocol³³ by dissolving CuBr and 2,2'-bipyridine in degassed water. Although a good amplification signal was obtained, the response was unstable and showed poor reproducibility. Most severely, the deposited material for signal enhancement could be removed by extensive rinsing, leading us to speculate that the signal may not be a result of polymerization but rather precipitation of deactivated catalyst when exposed to oxygen under the condition of inline flow injection analysis.

It is well documented that the ATRP process is sensitive to air since oxygen can cause irreversible oxidation of the transition metal catalyst and thus inhibit polymerization⁴⁴. Several deoxygenation procedures have been used for ATRP to maintain consistent polymer growth, which include the freeze-pump-thaw process⁴⁵ and placing the reaction

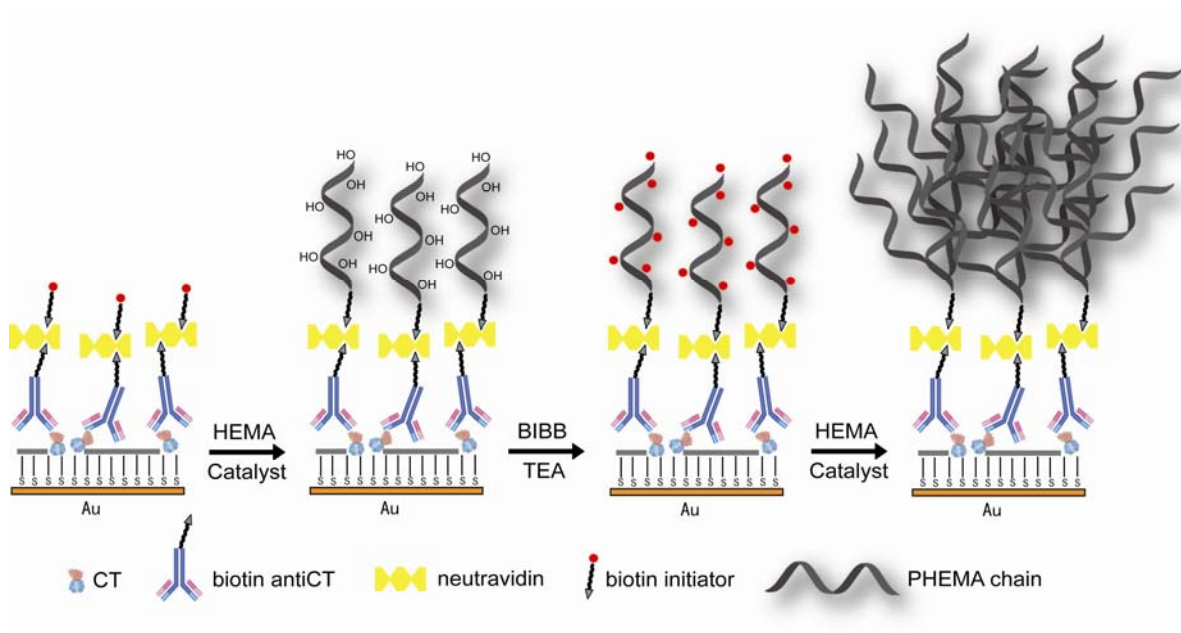


Figure 2.1. A cartoon representation of the biotinylated initiator coupled surface and consecutive two steps of in-situ surface ATRP reactions for SPR signal amplification.

system in a container under nitrogen protection³⁴. Although effective, these procedures are labor-intensive and not practical for inline SPR detection. Other approaches include the use of transition metal complexes with higher oxidation states to generate the ATRP initiator in situ⁴⁶ and addition of deoxygen reagents such as ascorbic acid (AA)⁴⁴ and glucose⁴⁷. We found that the catalyst solution (CuBr:2,2'-bipyridine in a 1:2 molar ratio) that was prepared in degassed water and sealed in an eppendorf tube developed a grey blue precipitation after 30 minutes. However, with the addition of AA (CuBr:2,2'-bipyridine:AA in a 1:2:1.5 molar ratio), the catalyst solution showed the original dark brown color after a long period of time (supplement). We then further evaluated the use of AA in ATRP catalyst for flow injection inline SPR detection. Figure 2.2 shows the comparison of SPR sensorgrams with and without AA addition. Here neutravidin was covalently attached to the MUA functionalized surface, the biotinylated initiator was injected and incubated, followed by 20% HEMA monomer/catalyst solution. The significant signal increase right after injection was caused by refractive index change. Following the incubation period for polymer growth, the SPR signal increased substantially in both cases. However, without AA in the catalyst solution, the materials formed on the surface could be easily rinsed off by PBS buffer, and the overall SPR signal increase was insignificant as compared to its original level (Figure 2.2a). By contrast, with AA in the catalyst solution, there was no visible precipitate formed on the surface, and a large SPR signal increase (0.27 degrees) was observed after even extensive rinsing (Figure 2.2b), demonstrating an effective in situ polymer growth in an FIA setting.

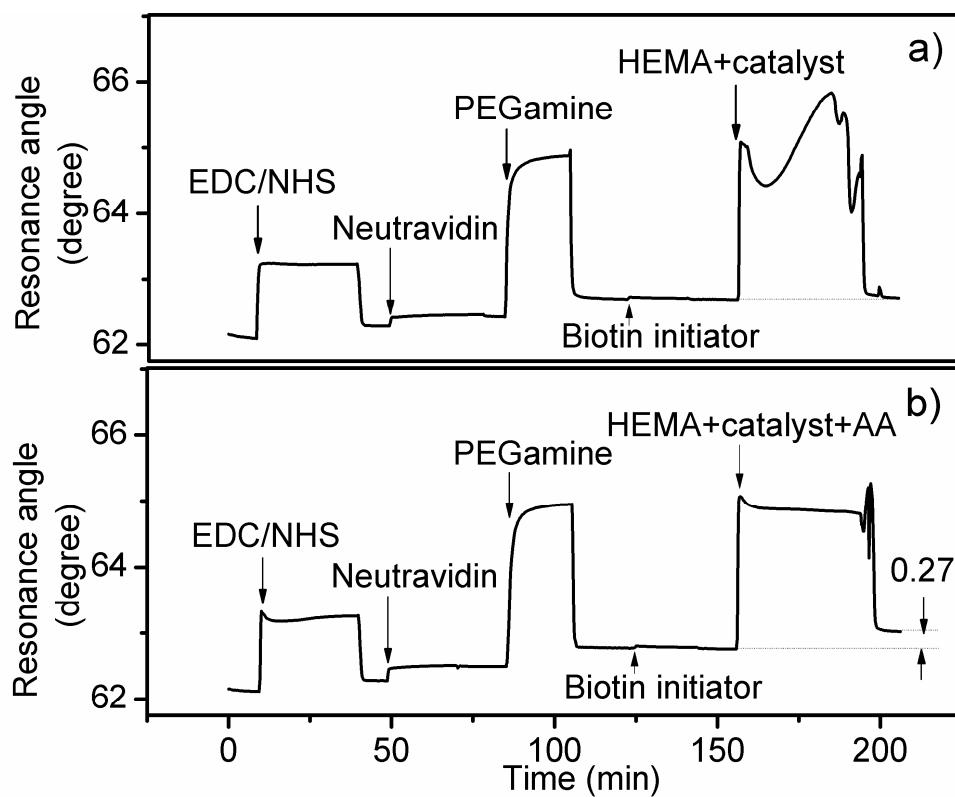


Figure 2.2. SPR sensorgrams for the covalently bound neutravidin-biotin initiator surface showing ATRP with HEMA monomers in the absence a) and presence b) of oxygen-suppression reagent ascorbic acid in the catalyst mixture.

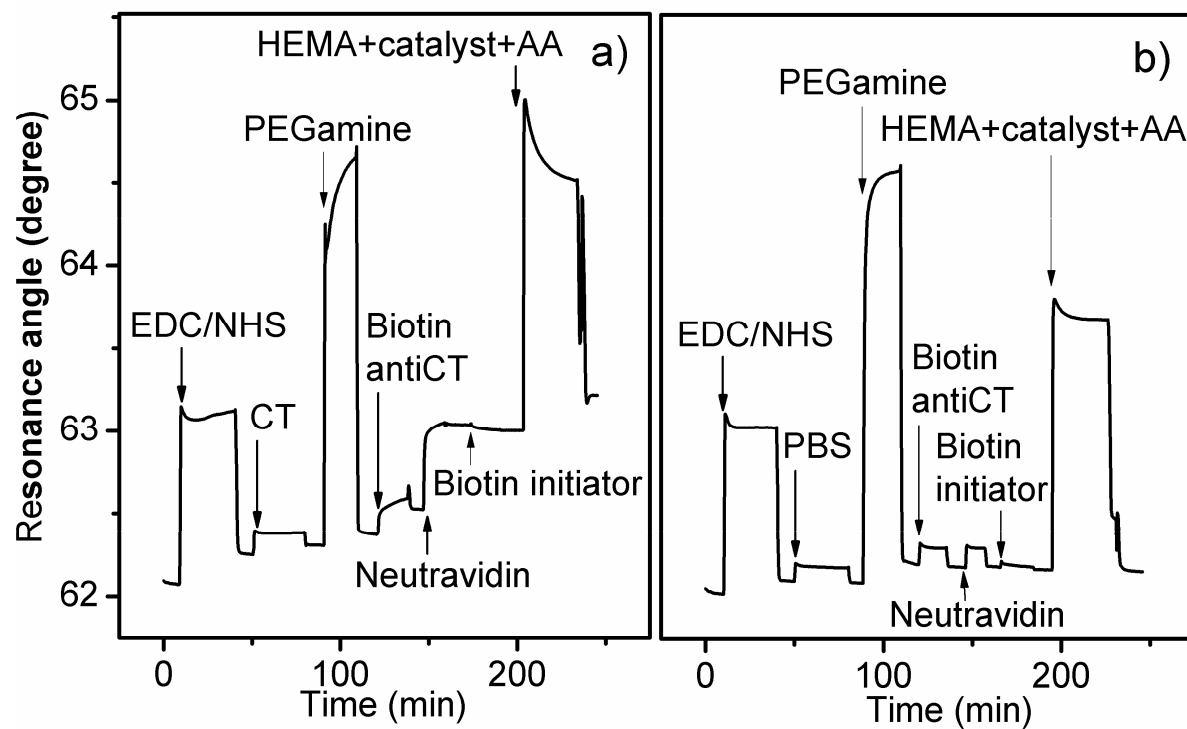


Figure 2.3. SPR sensorgrams for a) immunoassay of $2.92 \times 10^{-12} \text{ mol/cm}^2$ CT with ATRP amplification and b) the control channel.

When applying ATRP to CT detection and amplification, the HEMA/catalyst solution with AA in a 5:1 volume ratio was injected into the SPR flow-cell after biotin-initiator coupling. Figure 2-3 showed the results with 1.2 μ M CT used for surface immobilization. PHEMA grew on the surface during a 30 minutes incubation, resulting in an angle shift of 0.22 degrees after PBS rinsing, much larger than the CT attachment alone (0.06 degrees) (Figure 2.3a). In the control channel, buffer was injected instead of CT under the same condition, followed by biotin antiCT, neutravidin, and biotin initiator incubation, and ATRP. There was no measurable SPR signal change either for biotin antiCT and neutravidin, or for HEMA (Figure 2.3b), demonstrating good specificity of the polymer growth and successful suppression of nonspecific adsorption of the catalyst.

Although SPR signal amplification was achieved with biotinylated-initiator/HEMA, the extent of amplification was not very high, insufficient for ultra-sensitive SPR detection. It seems the surface concentration of initiator is relatively low in this case as compared to self-assembled monolayer of initiator-coupled small molecules⁴⁰. Furthermore, the ATRP process only generates localized, linear polymers from the sparsely present initiator spots, which can not provide substantial SPR signal enhancement. It has been observed that high yield polymer growth usually requires high surface initiator concentration and long reaction time (hours)³³⁻³⁵. Unfortunately, hours of incubation time for polymer growth is unsuitable for inline based FIA study. Alternatively, formation of hyper-branched polymer molecules can be pursued to obtain substantial materials on the surface⁴⁸. It is known that initiator precursor could react with hydroxyl groups or amine groups, and generated hyperbranched polymers via ATRP^{49,50},

which has been used for direct visualization of low concentration target³⁹. Using the hydroxyl groups of the original PHEMA chain as a starting point, we decided to introduce a second ATRP reaction to generate hyperbranched polymer for CT signal amplification. To do this, as illustrated in Figure 2.1, the PHEMA was activated with BIBB which converts the numerous hydroxyl moieties on the PHEMA to bromo groups that can subsequently serve as sites for PHEMA hyper-branches to grow. The resulting higher initiator density would offer high polymer growth efficiency and thus considerable increase in SPR angle shift. It should be mentioned that in addition to hydroxyl groups, neutravidin amine groups could also participate in the coupling reaction, but the more numerous side chain hydroxyl groups and relatively more exposed positions make them more accessible to 2-bromoisobutyryl in the initiator conversion step. After reaction with 2-bromoisobutyryl bromide and incubation with HEMA/catalyst for 30 minutes and PBS rinsing, resonance angle shifted substantially with 3.89 degrees of signal obtained for the bound CT (Figure 2.4a). The polymerization actually resulted in a considerable change of surface optical property, discernible to the naked eye. The control was performed under the same conditions in the absence of CT. Since there was no formed primary linear PHEMA or neutravidin in the first ATRP step, there was no position available for 2-bromoisobutyryl bromide to couple to. The second step ATRP only showed a very small signal shift (Figure 2.4b), demonstrating low background signal and abundant room for lowering the detection limit. Additional control was performed with biot-IgG, which is not specific to surface immobilized CT. The result showed negligible angular shift after two-step ATRP, further confirming the high specificity of the in-line polymer growth.

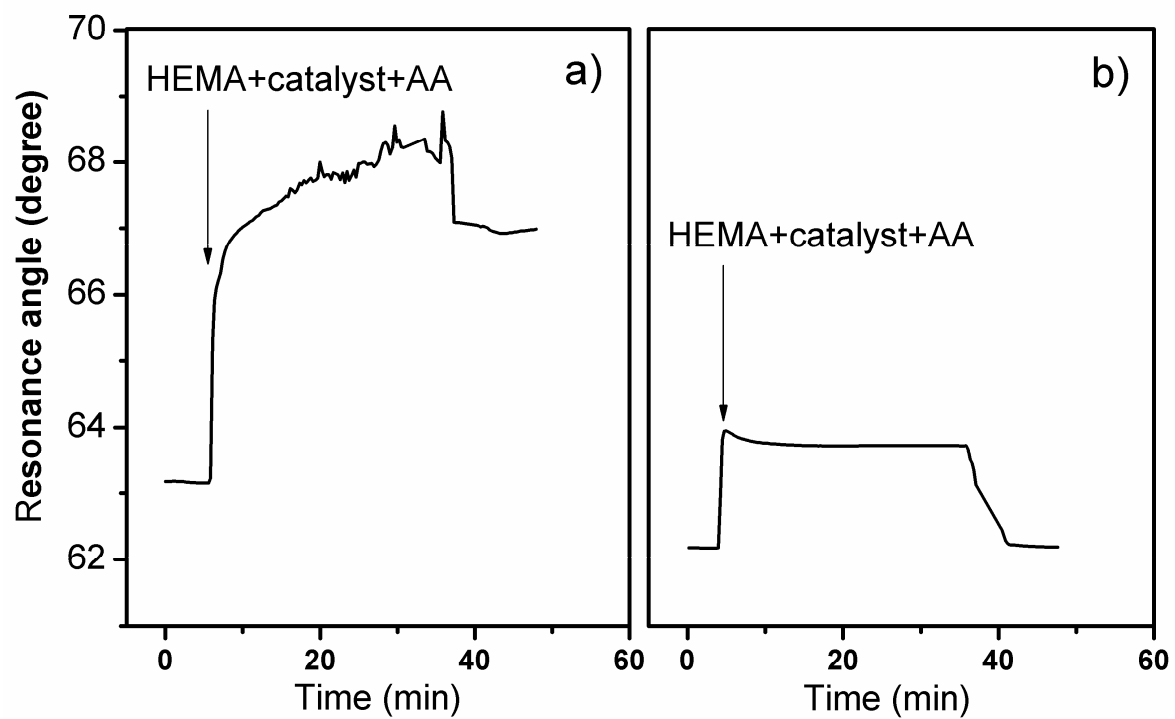


Figure 2.4. SPR characterization of the second step ATRP amplification for a) CT immobilized surface and b) the control surface without CT.

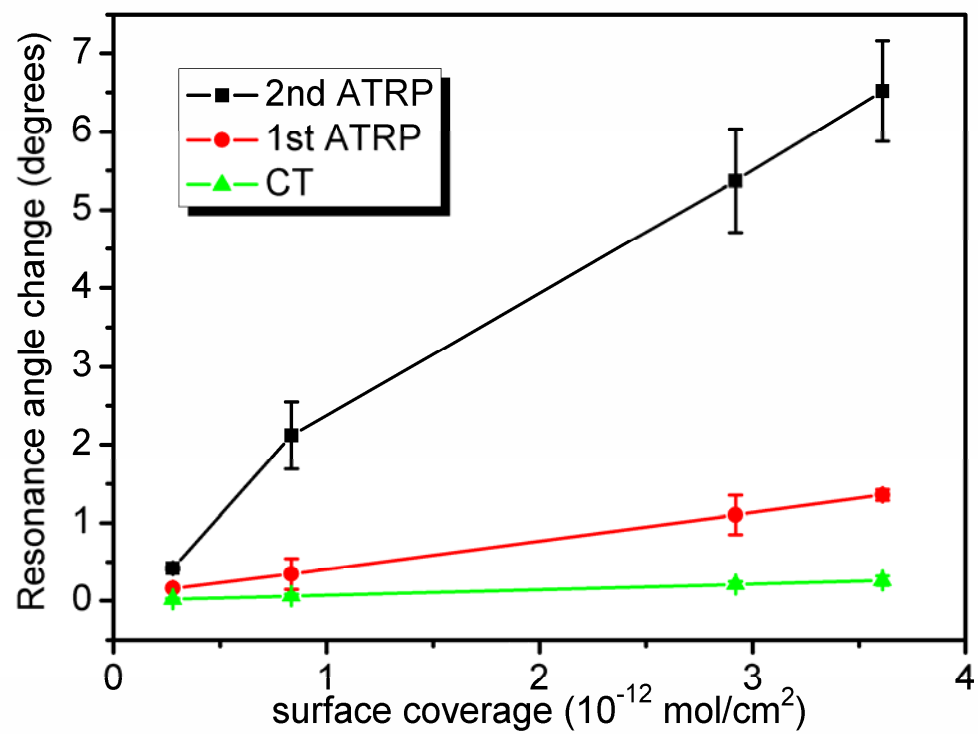


Figure 2.5. Comparison of SPR responses for direct CT coupling ($\blacktriangle$), after first ATRP amplification ($\bullet$), and after second ATRP amplification ($\blacksquare$).

2.3.2 Comparison of amplification effect for direct CT attachment, 1st step ATRP amplification, and 2nd step ATRP amplification

In order to quantify the amplification efficiency, SPR angular shifts for CT attachment, first step ATRP amplification, and second step ATRP amplification were compared. A series of CT-modified surfaces were prepared with variable solution phase CT concentrations. To best describe the amount of immobilized CT, a surface coverage was used instead of solution concentration. The CT surface coverage was determined according to Jung's formula⁵¹ using SPR angular shift, protein refractive index of 1.6⁵² and the bulk density of protein of 1.43g/cm³⁵³. Figure 2.5 shows the resonance angular change under various conditions versus the amount of CT attached. The plot range is from 2.8×10^{-13} to 3.6×10^{-12} mol/cm², as the angular shift of CT immobilization was difficult to accurately quantify when the surface coverage was lower than 2.8×10^{-13} mol/cm². Above 3.6×10^{-12} mol/cm², the second step ATRP was so efficient that the film thickness exceeded the effective decay length of the evanescent field of surface plasmons, leading to saturated signals. From Figure 2.5, both ATRP steps enhance SPR signal for CT, exhibiting a similar tendency of signal increase with respect to CT surface coverage. But magnitude of enhancement differs substantially. For instance, at 3.61×10^{-12} mol/cm², first step ATRP amplification increases CT signal by ~ 5 times, whereas the two steps ATRP amplification gives CT signal increase by about 25 times, which is better than many methods currently used for SPR signal enhancement^{24,25,28}, and therefore making it possible for high-sensitive detection of protein molecules.

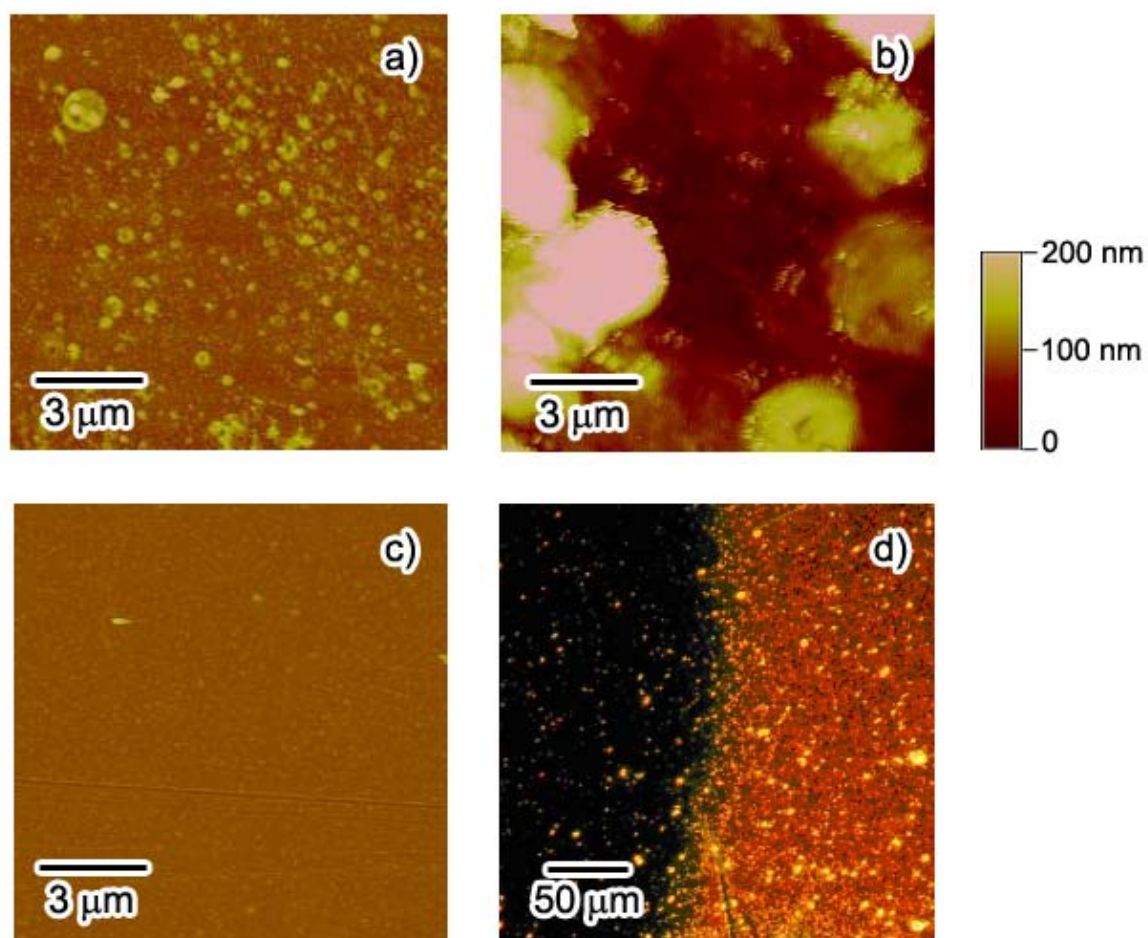


Figure 2.6. AFM images of the polymer films prepared with immobilization of $2.92 \times 10^{-12} \text{ mol/cm}^2$ of CT. a) after the first step ATRP; b) after the second step ATRP; c) the control surface without CT immobilization, and d) shows the microscopic image of the polymer film prepared with $3.61 \times 10^{-12} \text{ mol/cm}^2$ of CT after the second step ATRP.

2.3.3 Morphological characterization of ATRP polymer films

The inline formation of hyper-branched PHEMA was characterized by a number of methods including AFM and optical microscopy. AFM is capable of providing useful topographic information of the polymer branches in the nanometer scale. Figure 2.6a shows the surface feature of the PHEMA film after first step ATRP by AFM. At a surface coverage of 2.92×10^{-12} mol/cm² for CT, the linear polymer chains show rough surface property on some areas due to sparse initiator binding site. After second step ATRP, polymer islands composed of films with thickness above 100 nm and average feature size in the micrometer range were observed (Figure 2.6b). The hyperbranched PHEMA after second ATRP substantially expended the polymer coverage, but we also observed some thinner polymer sheets with micrometer-range intervals, possibly because the localized, short linear PHEMA from the first ATRP could not cover the entire surface and therefore limits the polymer growth in the voids. Previous work showed similar polymer morphology by AFM³⁹ with smaller polymer islands and interval distances, possibly due to the size difference between the DNA target in their research and protein CT used here. For comparison, the surface is quite flat and featureless in the absence of CT after two-step ATRP, indicating little polymer growth nor nonspecific adsorption (Figure 2.6c).

Direct inspection of the substrate confirmed the formation of a much thicker polymer layer as the optical quality of the surface changed. An optical image, obtained on the boundary of the flow cell after the ATRP reaction, clearly showed the difference (Figure 2.6d). The right bright area indicates the polymer growth, which was visible to naked eye, while the left dark area shows relatively clean background for the bare gold surface. The

surface coverage of CT in Figure 5d is 3.61×10^{-12} mol/cm², which yielded 6.53 degrees of angular shift after two-step ATRP. The hyperbranched PHEMA film has a clear edge line, showing a substantially large surface density and thickness for areas where CT is attached.

2.3.4 Effectiveness of surface passivation on background suppression

There are two key factors in the ultra-sensitive detection of protein molecules with SPR: amplification of the resonance angular shift and surface passivation. Suppression of nonspecific adsorption is of great importance to this SPR-based amplification method as trace amount of adsorbed species will generate very high signal after the amplification step. Hydrophilic polyethylene glycol is a material known to have a low interfacial free energy, and is capable of resisting nonspecific adsorption of biomolecules including protein⁵⁴ and DNA^{55,56}. In this work, PEGamine and other surface passivation agents were compared for their effectiveness in passivating the surface that meets the requirement for an amplification method. The carboxyl acid activated Au substrates were treated with PEGamine, 1 mg/mL BSA, and 10% ethanolamine solution, respectively, followed by exposure to 0.25 mg/mL neutravidin and a 20% HEMA solution. The results are summarized in Figure 2.7. The BSA-passivated surface showed a relatively significant amount of neutravidin adsorption due to protein interaction and a noticeable extent of HEMA nonspecific adsorption. Slight blocking improvement was observed for ethanolamine-coated surface against neutravidin nonspecific adsorption. However, ethanolamine is not effective as a passivating reagent to reduce HEMA nonspecific adsorption, because side chain hydroxyl groups of HEMA may stick to hydroxyl terminal

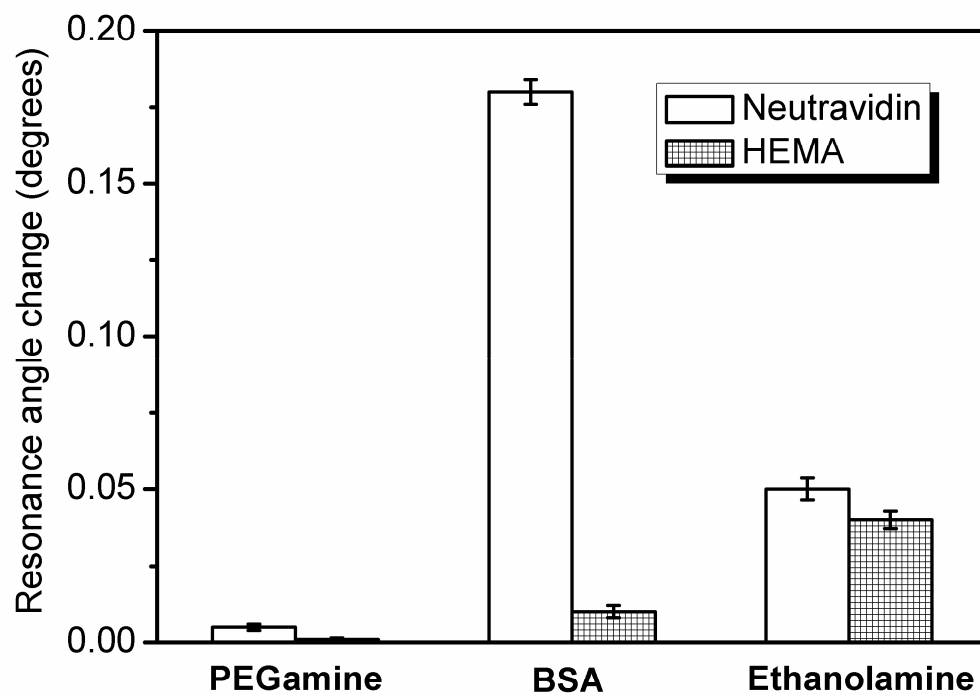


Figure 2.7. SPR angular shift in response to nonspecific adsorption of 0.25 mg/mL neutravidin (blank) and 20% HEMA (grid) on differently passivated Au substrates.

surface, which has been confirmed by previous work⁵⁷. The PEGamine-passivated surface shows excellent suppression of nonspecific adsorption from both the hydrophilic HEMA monomer and hydrophobic neutravidin. After a 10 min incubation for neutravidin and 30 min incubation for HEMA followed by PBS rinsing, SPR angular shift was negligible as compared to nonspecific adsorption on BSA and ethanolamine-passivated surfaces, making PEGamine an effective background suppression reagent for this polymer-based amplification method.

In addition, biotin-binding proteins have been screened and compared for their ability to decrease nonspecific adsorption on the PEGamine sublayer. Both avidin and neutravidin are commonly used biotin-binding proteins containing four available binding sites, and they share the same major structure except for the deglycosylated character of neutravidin, which is responsible for its near-neutral isoelectric point (pI). Avidin's high pI leaves it positively charged in PBS at pH 7.4, which lead to some degree of nonspecific adsorption on the PEGamine- passivated surface, as measured with an angular shift of 0.22 degrees after ATRP amplification. Neutravidin, on the other hand, is negatively charged at pH 7.4 and appears to not adhere to the PEGamine covered surface. It showed negligible SPR signal change after 10 minutes incubation and PBS rinsing, making it a suitable protein to be used for linking biotinylated initiator to the specific protein (CT) site.

2.3.5 Highly sensitive detection of protein toxins.

CT, an archetypal member of the AB₅ toxin family⁵⁸, is responsible for the deleterious effects of cholera infection, one of the most severe illnesses in developing countries. In

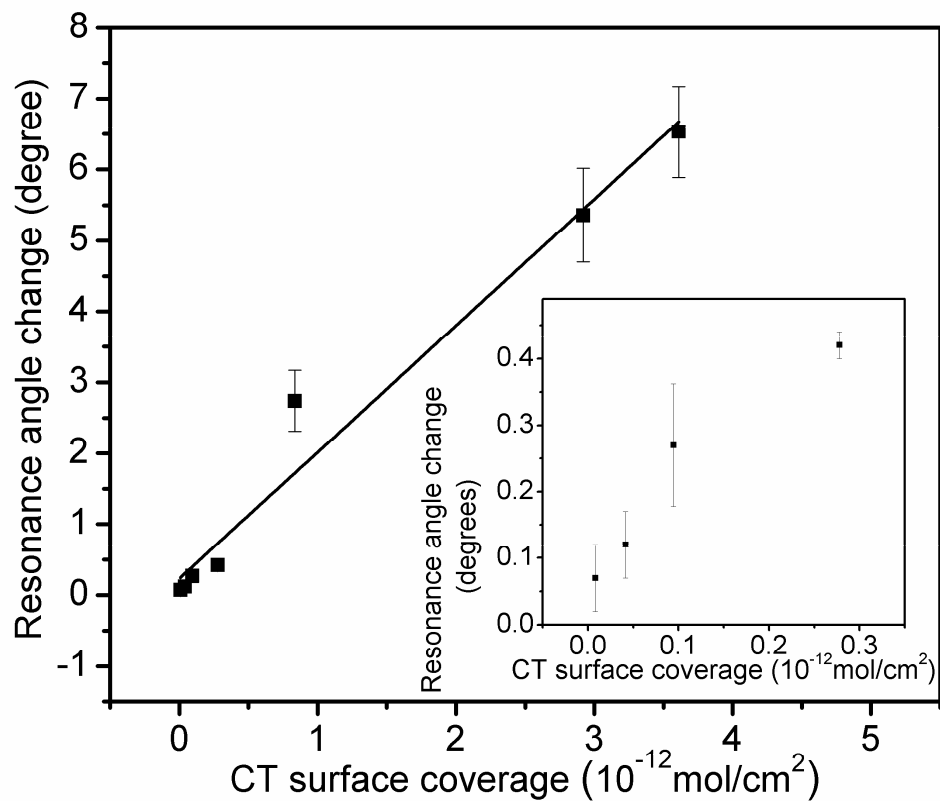


Figure 2.8. SPR response as a function of CT surface coverage after two steps ATRP amplification with biotinylated initiator and neutravidin. Inset displays the response for CT surface coverage lower than 8.35×10^{-13} mol/cm².

recent years, ultrasensitive detection of CT has drawn considerable interest because of the clinical relevance and excellent property as a model system due to well characterized interactions between CT and its cell surface ligand ganglioside (GM1)⁵⁹. In this work, we covalently immobilized CT to a self-assembled monolayer (SAM) of carboxyl acid terminal surface to demonstrate the principle of amplified protein detection with ultrahigh sensitivity. Figure 2.8 shows a plot of the SPR signal increase after two consecutive ATRP steps as a function of CT surface coverage. As the SPR signal for CT below the surface coverage of $2.78 \times 10^{-13} \text{ mol/cm}^2$ could not be directly measured, the lower surface coverage of CT in the plot was determined with extrapolation of the curve obtained with SPR measurements after neutravidin attachment. The ATRP amplified signal shows a linear correlation to the surface coverage of CT from $8.23 \times 10^{-15} \text{ mol/cm}^2$ to $3.61 \times 10^{-12} \text{ mol/cm}^2$ ($R^2=0.9219$). Even at $8.23 \times 10^{-15} \text{ mol/cm}^2$ (the concentration of CT used in immobilization is 59 pM), the immobilized CT generates discernible SPR signal change after ATRP amplification, which is much lower than most previously reported CT detection including a layer-by-layer SPR amplification⁶⁰ and colorimetry⁶¹. Inset in Figure 2.8 shows data and error bar for surface coverage less than $2.78 \times 10^{-13} \text{ mol/cm}^2$. Using the 3 S/N principle, the detection limit was determined to be $6.27 \times 10^{-15} \text{ mol/cm}^2$, which is almost 150 times more sensitive than the method with DOPC/GM1 membrane⁶². Taking the flow cell area into calculation, the absolute quantity of detection limit is 2.19 fmol, which is comparable to highly sensitive electrochemical and fluorescence methods⁶³⁻⁶⁵.

2.4 Conclusions

We report here a novel SPR signal amplification strategy for highly sensitive detection of proteins based on in-situ surface initiated ATRP reactions. Biotinylated initiators for poly(hydroxyl-ethyl methacrylate) are used with neutravidin for their specific attachment to the targeted protein and subsequent initiation of polymer growth is attained. Two consecutive ATRP processes are realized on the protein sites through converting the linear PHEMA side chain hydroxyl groups from the first step ATRP into new initiators for a second step ATRP. This practice substantially enhances the polymer yield on the surface, and has efficiently shortened the reaction time. The immobilized CT has been detected in the range of 8.23×10^{-15} to 3.61×10^{-12} mol/cm² and a detection limit as low as 6.27×10^{-15} mol/cm² (2.19 fmol) has been obtained.

To realize inline polymer growth under ambient conditions, addition of ascorbic acid to the catalyst solution as deoxygen reagent was firstly applied and it proved to be satisfactory for flow-injection based SPR detection. PEGamine has also proven to be an excellent reagent to passivate the sensing interface where nonspecific adsorption of both hydrophilic HEMA monomer and hydrophobic neutravidin are effectively suppressed. Given that a large number of biotinylated antibodies are commercially available as well as coupling kits for protein biotinylation, this ATRP based amplification method can be applied as a universal strategy to highly sensitive detection of nearly all proteins and peptides through flow injection analysis by SPR. Future work will focus on optimizing the immobilization density of initiators and applying the current system to microarray analysis with SPR imaging for high-throughput screening of complex biological systems.

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Chapter 3. Detection of Membrane-Binding Proteins by Surface Plasmon Resonance with an All-Aqueous Amplification Scheme

3.1 Introduction

The cell plasma membrane constitutes a critical platform for diverse biological processes such as ligand recognition, drug action, vesicle fusion, endocytosis and pore-formation by membrane-active peptides and proteins. The lipid-protein interaction is one of the most important interactions in biology, which is responsible for mediating numerous signal transduction pathways and regulating cellular functions. To better understand the lipid associated biological processes, supported lipid bilayer membrane (SBM), which consists of a phospholipids bilayer membrane structure on a solid surface, has been constructed to structurally mimic cell membrane for a broad range of investigations in combination with spectroscopic and microscopic techniques¹⁻⁵. SBMs are typically generated from lipid vesicles fusion on hydrated surfaces, including glass¹, hydrophilic PDMS², long-chain alkanethiol SAMs sublayer³, protein “blanket”⁴, and polyion “cushions”⁵. As an important biomimetic model, SBM retains the key physico-chemical properties of the cell membrane, and provides substrates for cell culture^{6, 7}, aqueous phase separation and protein incorporation⁸. Ionic conductance change has been measured through ion channels on SBM to study ion channel protein behavior on SBM⁹, and electrophoresis has been performed on SBM that separates

membrane-bound species within a native-like environment while maintaining its native shape and orientation¹⁰. Most recently, nanostructure SBM has been designed for biosensing microarrays^{11, 12}.

Varied methodologies have been developed to monitor recognition process on lipid membranes. Atomic force microscopy (AFM) offers spatial resolution (~1 nm) allowing for visualization of supramolecular assemblies of membrane proteins, structural transformation of membrane-embedded species, and local changes in fluidity and elasticity within the membrane¹³. Biomimetic vesicles coupling with advanced materials, such as carbon nanobute¹⁴, polydiacetylene (PDA)¹⁵, and fluorescent dyes¹⁶, have been used as platforms for biosensing operations. In recent years, surface plasmon resonance (SPR) has become an attractive approach for quantitative examination of lipid-protein interactions¹⁷. An oval glass cell with gold coating on one side has been used for simultaneous SPR and QCM measurement of the vesicle adsorption and supported phospholipids bilayer formation¹⁸. We have developed a hydrophilic surface with calcinated nanoscale silicate on gold that is ideal for generating “glass-based” lipid membranes for direct SPR measurement¹⁹. A tethered bilayer lipid membrane array has been reported for SPR imaging analysis of lipid-protein interactions²⁰. SPR has been employed for the study of phosphoinositide-protein interaction²¹, glycolipid-cholera toxin interaction²², and effect of pore-forming compounds on bilayer lipid membrane²³.

However, it is important to point out that direct SPR detection has met considerable limitation for ultralow concentrations of analytes since the normal detection limit of SPR lies in the nanomolar range. This has impeded further application of SPR in proteomics and disease diagnostics, especially for detection of trace proteins in complex biological samples where existence of a huge excess of abundant proteins presents a high technical challenge.

To develop a signal enhancement technique suited for supported bilayer membranes can be very challenging. It is well known that lipid membrane systems have poor stability and require a hydrated environment in order to function properly. In addition, many experimental conditions causes membrane damage, including alkalinity and high ionic strength of the working buffer, detergent washing, acidic and basic interferents, air exposure, and organics contact^{24, 25}. Therefore, amplification protocols using enzymatic reactions which involve ethanol rinsing²⁶ and enzymatic tetramethylbenzidine (TMB) precipitation that does not stay on the membrane²⁷, can not be directly applied to this fragile system. Several other amplification methods have been reported using mild reaction conditions such as bulky vesicles²⁸, mediated agglutination of GM1-functionalized liposomes²⁹, and layer-by-layer deposition of proteins²⁰, but the detection limit can only be extended to sub-nanomol or high picomol range.

We report here a new method that combines gold nanoparticle (AuNP) and *in situ* polymer growth for enhancing detection sensitivity to attomol level. AuNP has been employed as high mass labels for analytes on SBM to enhance the electrochemical binding signal³⁰ and improve the SPR detection sensitivity for biomolecules such as progesterone³¹, DNA and proteins³², but the enhancement is limited. Corn et al. has reported the use of poly(A) polymerase extension reaction coupled with poly(T)-coated AuNP to amplify signals from surface-bound miRNA³³. Our method relies on controlled *in situ* polymer growth by atom transfer radical polymerization (ATRP) reaction that is confined to the AuNP surface for specificity. Figure 3.1 shows the overall process that uses bacterial cholera toxin (CT) and membrane receptor monosialoganglioside GM1 as model system. Although surface ATRP has been used for a number of analytical applications for signal enhancement³⁴⁻³⁶, organic solvent is typically required for initiator conversion. This has practically eliminated the use of this approach to fragile bilayer membranes. To avoid conversion in hostile solvent, 16-mercaptohexadecanoic acid (16-MHDA) stabilized AuNP is pre-functionalized with initiator for poly(hydroxyethyl methacrylate) (PHEMA), and a biotin tag with PEG spacer is co-immobilized on AuNP to specifically localize the initiator-coated AuNP to the target protein via the biotinylated antibody (biotin antiCT) and an avidin bridge (Figure 3.1). Optical microscopy and atomic force microscopy (AFM) are used to characterize the surface property of the

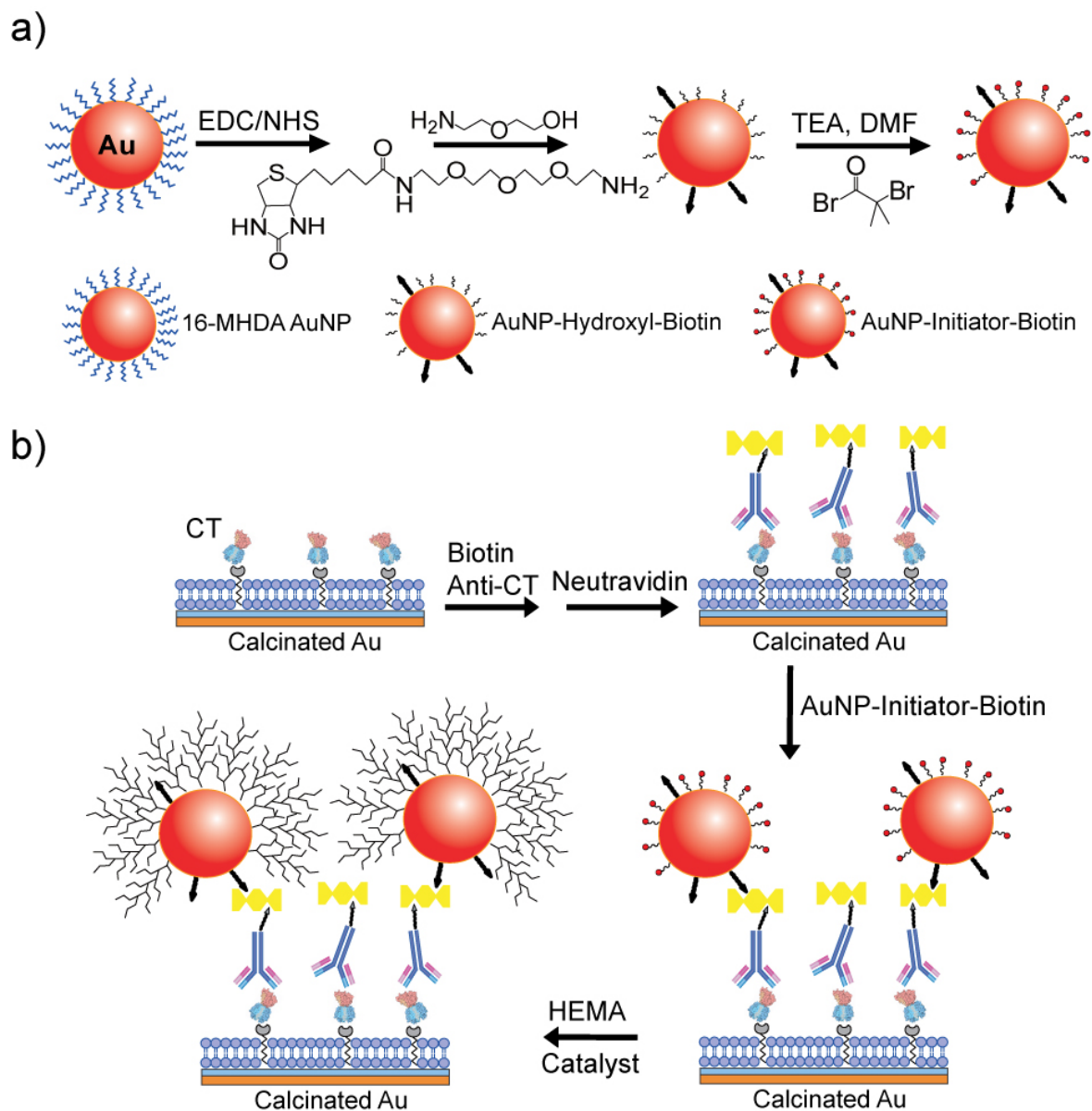


Figure 3.1. A cartoon representation of a) the AuNP surface modification with initiator and biotin, and b) *in-situ* AuNP coupling through biotin/avidin interaction and surface ATRP reaction for SPR signal amplification in CT detection.

particles and SPR chip. We have demonstrated the utility of this method for SPR detection of proteins in a complex biological sample with CT spiked in a blood serum sample and measured directly in high sensitivity without a separation step.

3.2 Experimental

3.2.1 Materials

Avidin and (+)-Biotinyl-3,6,9,-trioxaundecanediamine (BA) were obtained from Thermo Scientific (Rockford, IL). N-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl) -3-ethylcarbodiimide hydrochloride (EDC), 2-(2'-aminoethoxy) ethanol (AEE), 2-bromoisobutyryl bromide (BIBB), triethylamine (TEA), polyoxyethylene (20) sorbitan monolaurate (Tween 20), 16-mercaptohexadecanoic acid (16-MHDA), cholera toxin (CT), 3-mercaptopropionic acid (3-MPA), poly(allylamine hydrochloride) (PAH), 2-hydroxyethyl methacrylate (HEMA), CuBr, 2,2'-bipyridyl (bpy), and L-ascorbic acid (AA) were obtained from Sigma-Aldrich (St. Louis, MO). Sodium silicate was purchased from Fisher Scientific (Pittsburgh, PA). Biotinylated rabbit anti-cholera serum was from ViroStat (Portland, Maine). L- α phosphatidylcholine (PC) was purchased from Avanti (Alabaster, AL). Monosialoganglioside receptor (GM1) was obtained from Matreya (Pleasant Gap, PA). All proteins solutions were prepared in 20 mM phosphate buffered saline (PBS) (containing 150 mM NaCl, pH 7.4) unless indicated otherwise. Normal rabbit serum was

purchased from Invitrogen (Carlsbad, CA). The whole protein concentration is around 60 mg/mL, and diluted with PBS to 50% concentration (by volume) prior to CT spiking. Citrate stabilized AuNP solution with the AuNP particle size of about 20 nm was prepared according to the literature³⁷ and stored in brown glass bottles at 4 °C, which is stable for at least 1 month.

3.2.2 Preparation of AuNP-Hydroxyl-Biotin

AuNP was modified with hydroxyl and biotin groups according to a literature protocol³⁸ with some modification. Citrate stabilized AuNP dispersion with a concentration of 0.80 nM (determined by measuring absorbance at 520 nm and using an extinction coefficient of $1.25 \times 10^9 \text{ M}^{-1} \text{ cm}^{-1}$) was degassed with nitrogen gas. Tween 20 solution (1.82 mg/mL) was prepared in 10 mM phosphate buffer saline (PBS) at pH 7.0. 16-MHDA solution (0.50 mM) was prepared in degassed ethanol. Equal volumes (400 μL) of AuNP dispersions and Tween 20 solution were gently mixed and allowed to stand for 30 min for physisorption of Tween 20 to the AuNP surface. 400 μL 16-MHDA solution was added and allowed to stand for 3 hr for chemisorption to take place. The final mixture was centrifuged and resuspended in 10 mM PBS (with 1.82 mg/mL Tween 20, pH 7.0) for three times (30 min at 15700 g).

After the final centrifugation step, the 16-MHDA modified AuNPs were reacted with a mixture of freshly prepared 50 mM NHS and 200 mM EDC solution (10 mM PBS, pH

7.0) for 5 min. After washing with 10 mM PBS (with 1.82 mg/mL Tween 20, pH 7.0) (5 min at 15700 g), the remaining NHS ester-alkanethiol modified AuNP were reacted with a freshly prepared aqueous solution of AEE (22 mM) and BA (2.4 mM) for 20 min. After washing with 10 mM PBS (with 1.82 mg/mL Tween 20, pH 7.0) (5 min at 15700 g), the resulted AuNP-Hydroxyl-Biotin was stored at 4 °C.

3.2.3 Preparation of AuNP-Initiator-Biotin

Initiators were converted from AuNP surface hydroxyl groups via direct coupling of BIBB. Nitrogen-dried AuNP-Hydroxyl-Biotin was reacted with 8 mM BIBB and 10 mM TEA in DMF for 30 min. After washing with 10 mM PBS (with 1.82 mg/mL Tween 20, pH 7.0) (5 min at 15700 g), the generated AuNP-Initiator-Biotin were stored at 4 °C.

3.2.4 Preparation of calcinated gold chips

SPR gold substrates were fabricated with deposition of a 2 nm chromium adhesion layer, followed by e-beam deposition of 46 nm gold layer onto pre-cleaned glass slides. The nanoglassified layers were constructed based on a previously reported layer-by-layer (LbL) protocol³⁹. Clean gold substrates were immersed in 10 mM 3- MPA ethanol solution overnight to form a self-assembled monolayer. After extensive rinsing with ethanol and nanopure water and drying with nitrogen, modified gold substrates were alternated dipped into sodium silicate solution (22 mg/mL, adjusted to pH 9.5) and PAH solution (1 mg/mL, adjusted to pH 8.0) for 1 min to form a LbL assembly structure, with

sufficient water rinsing between layers. This dipping procedure was repeated eight times to build up a multilayered chip, followed by calcination in a furnace by heating to 450 °C at a rate of 17 °C per min and subsequent cooling to room temperature 4 hours later.

3.2.5 Vesicle preparation

Vesicle solutions were prepared from stock solution of lipids in chloroform. 180.5 μ L PC lipid stock solution (5 mg/mL) and 39.1 μ L GM1 stock solution (2.5 mg/mL) (95/5 mol% PC/GM1) were transferred to a small vial and the organic solvent was removed with N₂ to form a dry lipid film on the vial wall, which was then rehydrated with 1 mL 20 mM phosphate buffer (150 mM NaCl, pH 7.40) to have a lipid concentration of 1 mg/mL. The resuspended lipids were probe sonicated for 20 min, followed by centrifugation at 8000 rpm for 6 min to remove any titanium particles released from the probe tip. The supernatant was then extruded with 11 passes through a polycarbonate membrane with pore size of 100 nm to ensure formation of small unilamellar vesicles. The solution was then incubated at 4 °C for at least 1 h before use.

3.2.6 SPR measurement and signal amplification with AuNP-PHEMA

A dual channel SPR spectrometer NanoSPR-321 (NanoSPR, Addison, IL) with a GaAs semiconductor laser light source (λ =670 nm) was used for all SPR measurements. The device comes with a high-refractive index prism (n =1.61) and 30 μ L flow cell. Surface interaction and modification were monitored using the angular scanning mode around the

minimum angle. After extensive rinsing with ethanol, DI water, and dried under a N₂ stream, the calcinated gold substrate was clamped to a flow cell on a prism. The mixed vesicle solutions (95/5 mol% PC/GM1) were injected into the flow cell and incubated for 1 h to allow vesicle fusion to take place and form bilayer membrane, which was followed by 10 min rinsing to completely remove the unused vesicles. Varied concentrations of CT in PBS were injected and incubated for 30 min to allow sufficient protein binding. The immunoassay was carried out with injection of 0.25 mg/mL biotin anti-CT and 20 min incubation. After the surface was rinsed for 10 min, 0.2 mg/mL avidin was injected and incubated for 25 min before running a 10 min rinsing.

Before the injection of AuNP-Initiator-Biotin, the running PBS buffer was changed to water to avoid colloid aggregation caused by salt effect. After 30 min incubation, the surface was rinsed with water for 10 min to remove nonspecific and unstable surface bound AuNP-Initiator-Biotin. The amplification with ATRP was conducted with an aqueous solution of 20% HEMA monomer and catalyst (CuBr-2,2'-bipyridine-AA in a 1:2:1.5 molar ratio). Ascorbic acid (AA) was added as oxygen suppressor to prevent catalyst from deactivation³⁴. The monomer and catalyst solution was purged with nitrogen for 30 min before injection to reduce the amount of O₂ present. After incubation for 30 min, the polymerization was stopped by removal of the HEMA/catalyst mixture with PBS buffer.

3.3 Results and Discussion

3.3.1 Functionalization of AuNP for ATRP polymer growth

The functionalization of AuNP is characterized by UV spectroscopy as the position of surface plasmon (SP) band of AuNP is sensitive to the local chemical environment and associated with particle-size distribution. After the modification of hydroxyl and biotin functional groups, a shift from 522 nm to 545 nm was observed. The presence of oligo(ethylene glycol) moieties in the biotin ligand chains (BA) provides surface shielding and prevents AuNP from aggregation. The existence of hydrophilic hydroxyl groups on AuNP surface further contributes to the dispersion of AuNP-Hydroxyl-Biotin in solution. The conversion of hydroxyl groups to initiators shifts AuNP absorption peak to 566 nm with noticeable band broadening, which is due to the loss of surface charges and subsequent reduction of repulsive electrostatic forces. Tween 20 was thus used as additive in PBS buffer solution for the resuspension of AuNP after each centrifugation steps, which can preserve the stability of AuNP after surface modification^{38, 40}.

The presence of biotin groups on the AuNP surface was first verified by solution reactions. Incubation of 0.80 nM AuNP-Hydroxyl-Biotin and 20 nM neutravidin led to aggregation and thus precipitation, while non-biotinylated AuNP-Hydroxyl remained well dispersed after the addition of neutravidin.

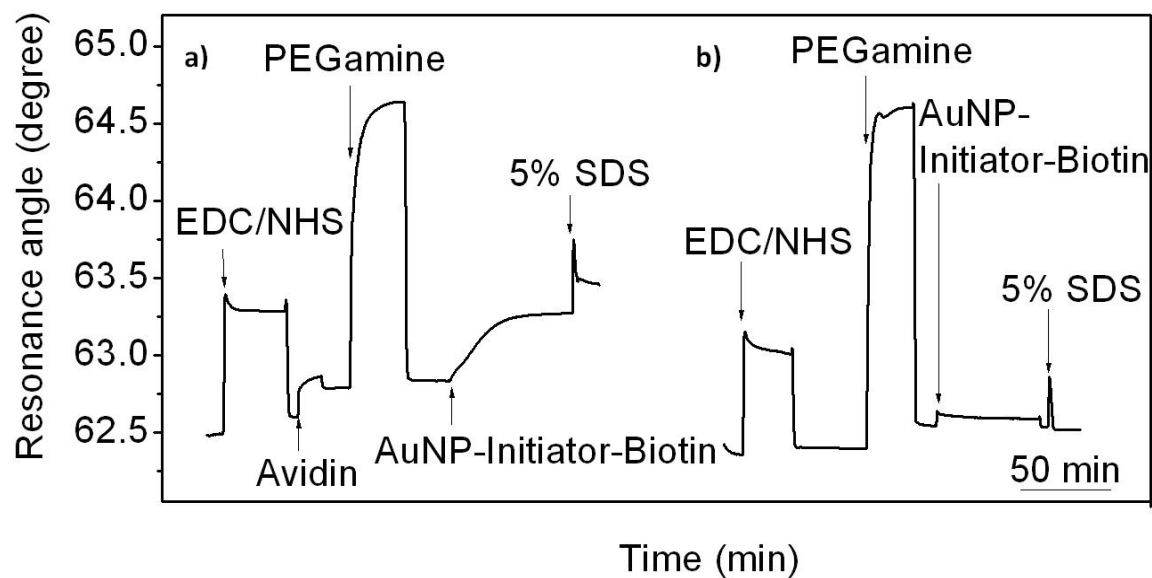


Figure 3.2. SPR sensorgrams for a) initiator and biotin modified AuNP binding to surface via biotin/avidin interaction and b) the control channel.

The capture of AuNP-Initiator-Biotin to the sensing surface was assessed by SPR using surface immobilized avidin for simplicity of the procedure (Figure 3.1). After the surface was blocked with PEGamine, AuNP-Initiator-Biotin was injected and incubated for 1 h. SPR signal increased by 0.44 degree after 10 min rinsing (Figure 3.2a). The binding of AuNP was stable and not affected by surfactant (5% SDS) rinsing, which is essential to the follow-up ATRP reaction. Control was performed in the absence of avidin, and there was no measurable signal increase upon AuNP-Initiator-Biotin incubation (Figure 3.2b), demonstrating good specificity for the AuNP-Initiator-Biotin to the avidin-functionalized surface.

To confirm the attachment of initiators to the AuNP surface, solution phase ATRP was first conducted by mixing AuNP-Initiator-Biotin with HEMA in the presence of the catalyst. After 30 min reaction under nitrogen protection, formation of poly(2-hydroxyethyl methacrylate) (PHEMA) on the AuNP surface shifted the surface plasmon band to 534 nm with a concomitant narrowing of the spectrum. The hydrophilic PHEMA coating increased AuNP dispersion with side chain hydroxyl groups. An absorption peak was observed around 300 nm, which is identified as a characteristic peak for UV spectrum of PHEMA⁴¹. The polymer coating on AuNP was also characterized using dynamic light scattering (DLS). Figure 3.3 shows the DLS result that the mean hydrated diameter of particles increased from 17 nm (citrate stabilized AuNP) to 215 nm

(AuNP-PHEMA) after ATRP, demonstrating successful formation of polymer shell around the nanoparticles. The particle size distribution also increased noticeably after polymer growth, which is similar to the previously published results^{42, 43}.

3.3.2 Signal amplification with AuNP and inline ATRP on bilayer membranes

The application of AuNP-PHEMA to SPR signal enhancement was demonstrated with bacterial cholera toxin and GM1 incorporated in supported PC bilayer membranes that were formed by direct fusion on the calcinated gold surface. As shown in Figure 3.1, the surface bound CT was recognized by biotinylated anti-CT, which provided a handle to link initiator-bearing nanoparticles to the analyte via an avidin bridge. Two consecutive steps contribute to SPR signal amplification: AuNP-Initiator-Biotin binding and localized ATRP polymer growth on the AuNP surface. Figure 3.4a shows the amplification effect with 120 nM CT. The small signal decrease before AuNP-Initiator-Biotin injection was due to buffer change, which was necessary for preventing AuNP aggregation and reduction in ATRP efficiency. After 30 min incubation when AuNP-Initiator-Biotin achieved sufficient binding on the surface, ATRP was initiated by the injection of HEMA/catalyst/AA solution in a 5:1 volume ratio. The growth of PHEMA shifted the resonance angle by 1.39 degree. Combined with contribution from AuNP binding (~1.00 degree), the angular shift was amplified substantially as compared to the direct CT binding (0.11 degree). In the control channel, buffer was injected instead of CT under the

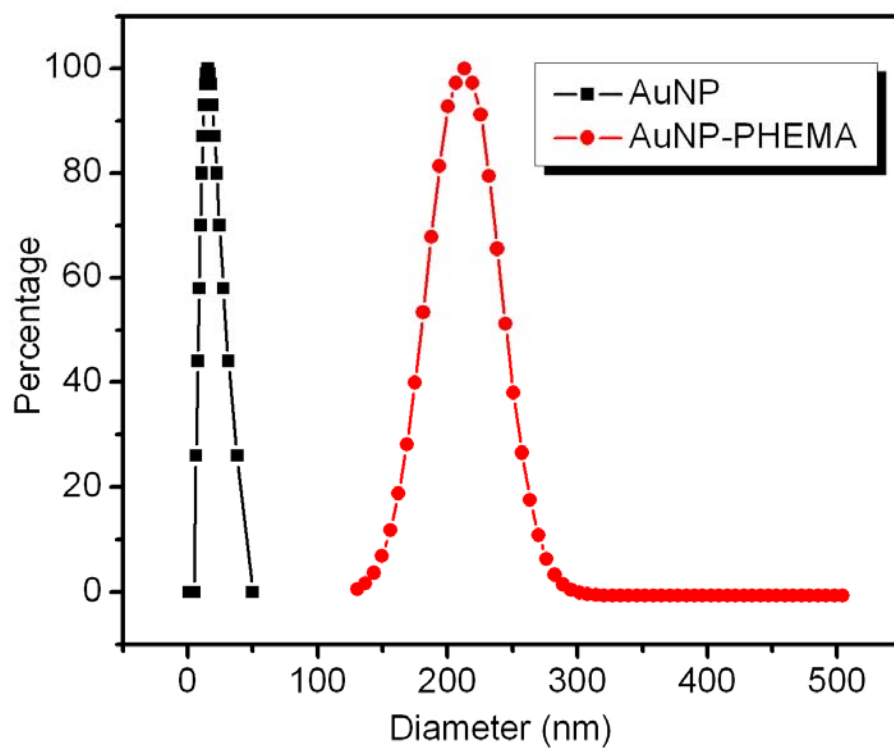


Figure 3.3. Dynamic light scattering measurements of AuNP (■) and AuNP coated with initiator and biotin after ATRP reaction (●).

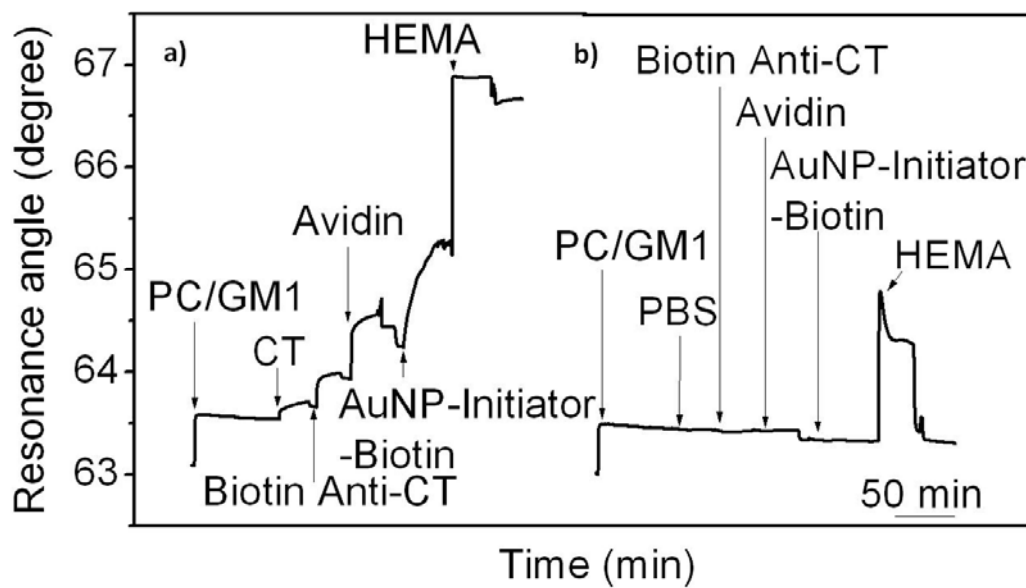


Figure 3.4. SPR sensorgrams for a) immunoassay of 120 nm CT on the PC/GM1 surface and its amplification with AuNP coupling and *in-situ* ATRP, and b) control surface in the absence of CT.

identical condition, which was followed by biotinylated anti-CT, avidin, AuNP-Initiator-Biotin, and HEMA/catalyst solution. Nonspecific binding signal was measured after incubation and surface rinsing. There was no detectable SPR signal change for biotin anti-CT, avidin, AuNP-Initiator-Biotin, or PHEMA (Figure 3.4b), demonstrating successful suppression of nonspecific adsorption by the supported membrane and excellent specificity of AuNP-Initiator-Biotin binding.

3.3.3 Comparison of signal amplification effect with direct CT binding, AuNP amplification, and AuNP-PHEMA polymer growth amplification

To quantify the amplification efficiency, SPR angular shifts for direct CT binding, AuNP amplification, and AuNP-PHEMA amplification were compared. Figure 3.5 compares a series of CT solutions measured with PC/GM1 bilayer membranes and SPR angular shifts for each amplification step. A plot range from 0.63 fM to 0.59 μ M was chosen for presentation as the amplification was so efficient that above 0.59 μ M the signal exceeded the detection range of the instrument. In the absence of the amplification, CT binding signal was very small at 0.59 μ M, which is consistent with literature results using direct SPR detection⁴⁴. AuNP alone could significantly enhance the signal and extend the detection limit to 59 pM, showing a 4 orders of magnitude improvement. *In situ* PHEMA polymer growth, however, had further markedly improved the detection limit to a new level. We have achieved a detection limit of 0.63 fM of CT using the AuNP

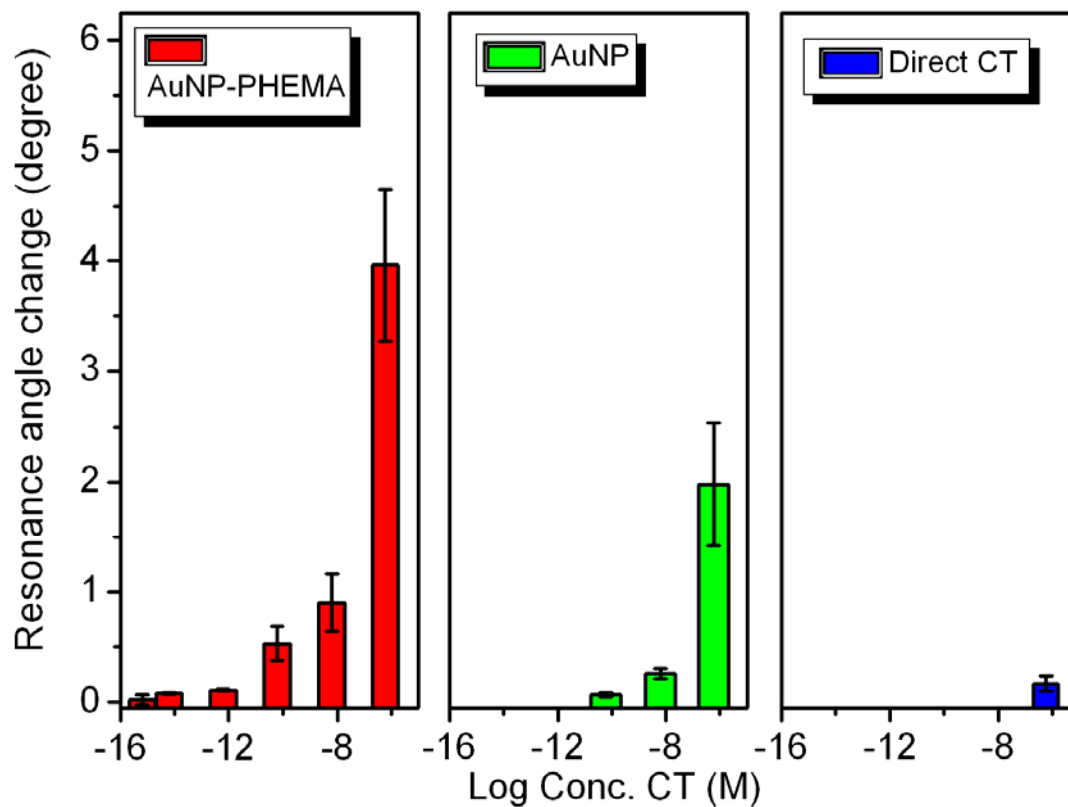


Figure 3.5. Comparison of SPR response of direct CT binding (blue), AuNP amplification (green), and AuNP/*in-situ* ATRP amplification (red) with a serial of CT concentration 5.88×10^{-7} M, 6.31×10^{-9} M, 5.89×10^{-11} M, 6.31×10^{-13} M, 6.31×10^{-15} M, 6.31×10^{-16} M.

and inline ATRP strategy, which is almost nine orders of magnitude lower compared to direct SPR detection. This result stems from highly effective polymer growth that filled the internal space among AuNPs and thus substantially increased the surface coverage, making it a desirable approach for ultrasensitive detection of membrane binding proteins.

3.3.4 CT quantitative detection on lipid membrane via AuNP-PHEMA SPR signal amplification

We then specifically investigated the response behavior of ultra-low concentrations of CT. Figure 3.6 shows the calibration curve of SPR signal as a function of CT concentration after AuNP-PHEMA amplification. The AuNP-ATRP amplified signal appears to show two segments of linear correlation to CT concentration. From 5.9×10^{-7} M to 6.3×10^{-8} M, the surface bound protein generated substantial AuNP binding and polymer growth, resulted in a much steeper calibration curve. This type of segmental calibration curve was also reported by other groups for IgG fluorescence detection^{45, 46}. Another linear range is from 6.3×10^{-8} M to 6.3×10^{-16} M ($R^2=0.9293$), which is more significant to ultrasensitive detection. SPR signal for 6.3×10^{-16} M CT was clearly obtainable at ~ 20 millidegrees, and this concentration is much lower than most previously reported CT detection methods including the layer-by-layer SPR amplification²⁰, colorimetric method⁴⁷, flow cytometry with fluorescently coded microspheres⁴⁸, and AuNP tracking on supported bilayer platform⁴⁹. Using 3 S/N cutoff,

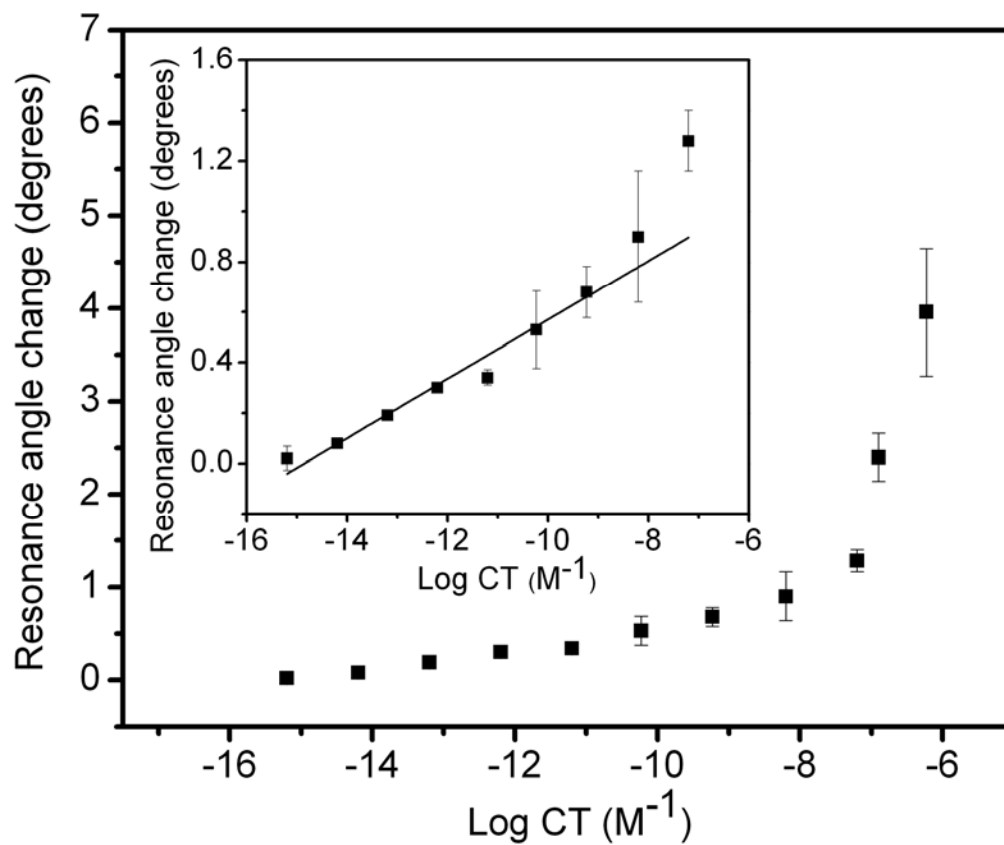


Figure 3.6. SPR response curve for CT detection using AuNP/ATRP amplification. Inset is the calibration curve with CT concentration lower than 6.3×10^{-8} M for emphasizing detection of low concentrations.

the detection limit was determined to be 1.6×10^{-16} M. Taking the flow cell volume into calculation, the absolute quantity of detection limit is 1.6×10^{-20} mol, which equals to ~9500 molecules in 100 μ L sample solution. This yields about nine orders of magnitude better detection than direct measurement, and six orders of magnitude lower than fluorescence based methods². The result is comparable to those by highly sensitive electrochemical detection with carbon nanotubes amplification⁵⁰, fluorescence-based flow injection immunoanalysis⁵¹, and capillary electrokinetic chromatography with multiphoton-excited fluorescence⁵².

3.3.5 Morphological characterization of AuNP and ATRP polymer films

The inline attachment of AuNP and formation of PHMEA were characterized by a number of methods including AFM and optical microscopy. Figure 3.7a shows the AFM images of the gold surface after AuNP attachment in the presence of 5.9×10^{-7} M CT. After inline PHEMA polymer growth, the average feature size increased to micrometer range and generated a relatively rough surface morphology (Figure 3.7b). Direct inspection of the substrates also confirmed the formation of PHEMA polymer layer, which changed the surface optical property. Figure 3.7c-d shows optical images obtained after ATRP amplification, suggesting the heavy influence by CT concentration. Incubation of 6.3×10^{-13} M CT caused 0.30 degree of angular shift after polymer growth. The formed PHEMA appeared to be packed loosely with a significant degree of uncovered area

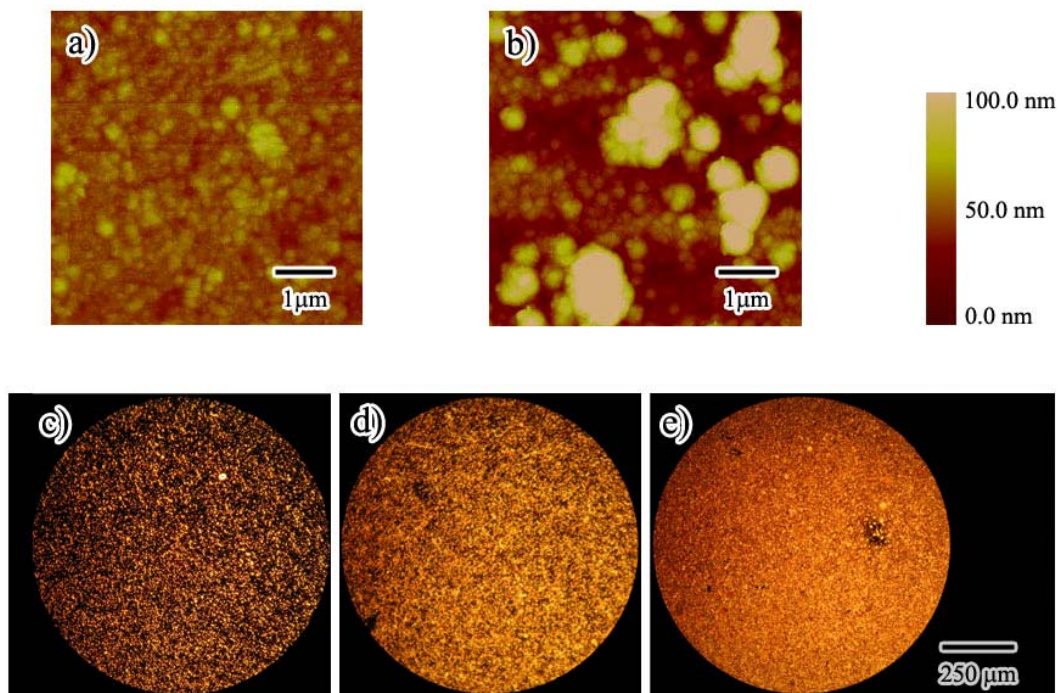


Figure 3.7. AFM images of a) the surface attached AuNP in the presence of 5.9×10^{-7} M CT and b) after *in-situ* polymer growth. Bottom are microscopic images of the AuNP-PHEMA films prepared with c) 6.3×10^{-13} M, d) 1.3×10^{-7} M and e) 5.9×10^{-7} M CT after AuNP/ATRP amplification.

(Figure 3.7c). When CT concentration increased to 1.3×10^{-7} M, the PHEMA coverage was very high, yielding 2.4 degree of angular shift and generating a soft stain in the detected area (Figure 3.7d). When 5.9×10^{-7} M of CT was used, it generated 3.96 degree of angular shift. The high efficient polymer growth substantially changed the surface refractive index, making the detected area clearly visible to naked eye (Figure 3.7e).

3.3.6 Detection of low concentrations of protein toxins in complex samples

The lethal dose for cholera toxin in human is relatively low ($LD_{50} = 250 \mu\text{g kg}^{-1}$), thus the ability to detect proteins sensitively from complex samples such as serum without the use of exogenous reagents or extensive separation procedure is desirable for many clinical/diagnostic applications. However, background interference remains a high challenge. Different strategies have been developed to suppress sample background, and well crafted surface chemistry has been sought after to diminish nonspecific adsorption⁵³. A common strategy used in immunoassay is to block the surface with bovine serum albumin (BSA) solution⁵⁴. In addition to blocking strategies, “non-stick” coating based on dextran^{55, 56} and oligo(ethylene glycol) (OEG)^{57, 58} has also been applied. Phospholipid bilayer membrane are very promising as coating material due to the native resistance to nonspecific protein adsorption^{59, 60}. We have previously reported a “membrane cloaking” method that selectively removes nonspecifically adsorbed proteins before final measurement by SPR⁶¹. Here we demonstrate the use of high amplification

efficiency and marked selectivity of the AuNP/ATRP strategy to boost signals specifically from the target protein, while leaving the nonspecific adsorption signal untouched, which can be easily discerned and subtracted. To validate the approach for real-world samples, we performed direct SPR detection of CT in a complex sample where 50% diluted rabbit blood serum were spiked with 1 μ M, 10 nM, 50 pM of CT. The results are shown in Figure 3.8 above and compared with those performed in PBS buffer. It should be mentioned that direct binding of CT spiked in serum solution can not be definitely measured for all three samples due to markedly high background. However, the amplification method developed here appeared to work effectively and specifically on target protein while the nonspecific adsorption signal remained flat even with injection of biotinylated anti-CT and avidin (Figure 3.8, bottom). As a result, as low as 50 pM CT can be detected from serum using AuNP/ATRP amplification. This concentration is a little high as compared to the measurement in PBS buffer, indicating that exposing to complex serum resulted in a decrease in sensitivity, which has been observed in previous report⁵³. We also noticed that complex biological samples have some degree of interference on amplification effect. Amplification signal in serum sample with 1 μ M CT was reduced to 63% of that in PBS buffer. As target concentration in serum was further decreased to 50 pM, amplification decreased to 32% of the signal obtained in the PBS buffer. Nevertheless, the method still effectively extended the detection limit six orders of

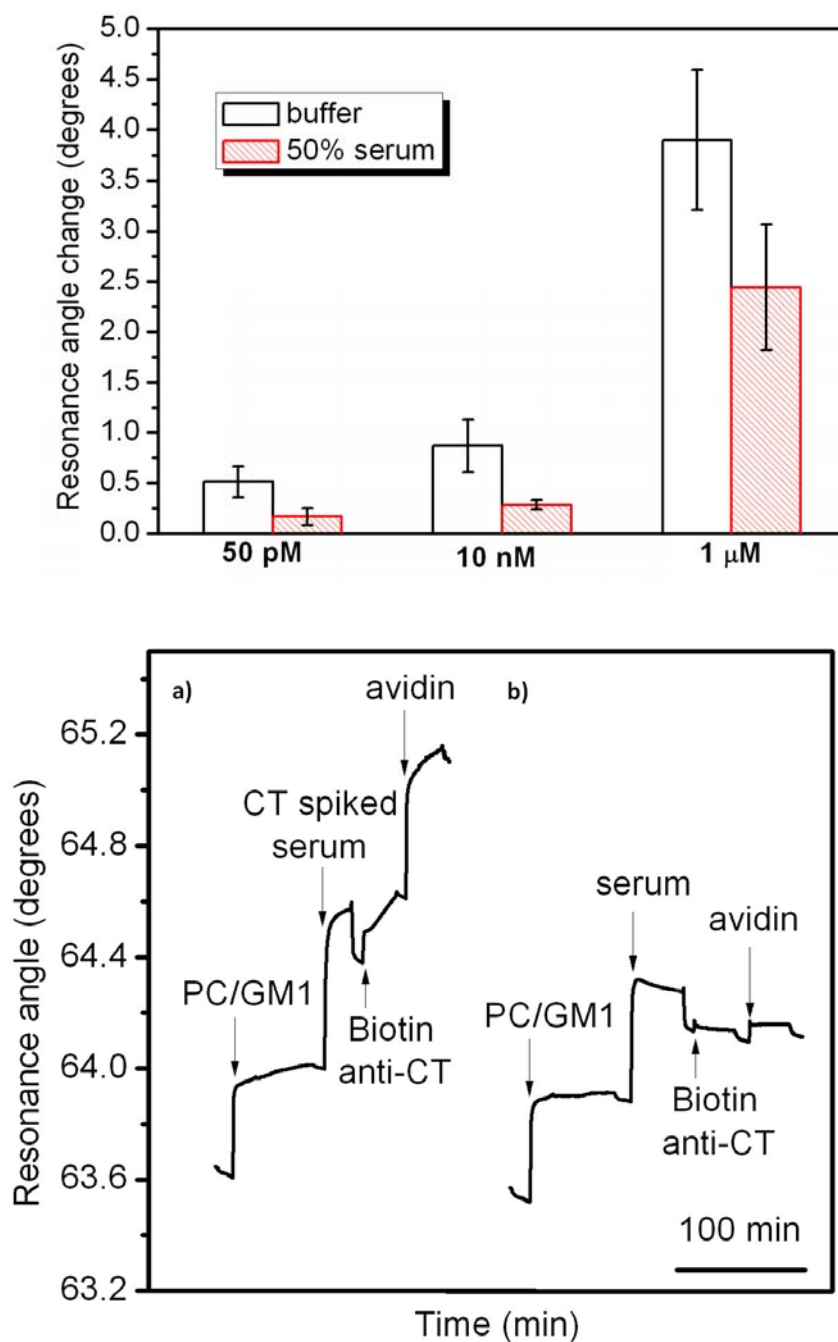


Figure 3.8. (Top) Comparison of SPR measurement of 1 μ M, 10 nM, 50 pM CT in PBS buffer and in 50% diluted rabbit serum after AuNP/ATRP amplification. (Bottom) SPR sensorgrams showing immunoassay and amplification on PC/GM1. a) CT-spiked 50% rabbit serum, and b) control with 50% rabbit serum only.

magnitude lower compared to direct CT detection in serum, and the signal was measurable even when CT concentration was lower than 50 pM. Clearly with the use of supported lipid membrane as sensing interface that has intrinsic property against nonspecific adsorption, this amplification method can provide high performance clinical assays with marked improved sensitivity and simple procedure for real biological samples.

3.4 Conclusion

We have reported a novel inline SPR signal amplification strategy for ultra-sensitive detection of proteins in an all aqueous environment based on AuNP-assisted ATRP reaction. AuNP was functionalized with long chain biotin tag and hydroxyl functional group, which allows initiator conversion before its attachment to target proteins on the lipid membrane.

Therefore, ATRP reaction can be conducted in a very mild condition, which makes the amplification strategy applicable for membrane binding events. PHEMA growth at the AuNP surface extensively increased surface mass and refractive index. As a result, the method extended the detection limit nine orders of magnitude lower compared to direct measurement on the same lipid membrane interface. The membrane bound CT has been detected in the range of 5.9×10^{-7} M to 6.3×10^{-16} M, and a detection limit of 1.6×10^{-16} M was achieved (equals to ~ 9500 molecules in a sample volume of 100 μ L). To examine the

amplification effect for complex samples, rabbit serum spiked with various concentrations of CT was investigated. We demonstrated a six orders of magnitude lower detection limit after AuNP/ ATRP amplification as compared with direct measurement from serum sample. Given that a large number of biotinylated antibodies are commercially available and coupling kits for protein biotinylation, this amplification method can be applied as a universal strategy for ultra-sensitive detection of proteins and peptides. Future work will focus on applying the current system to microarray analysis with SPR imaging for high-throughput screening of complex biological systems.

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Chapter 4 Highly Sensitive Detection of Bacterial Protein Toxin on Patterned Microarray by SPR Imaging with Nanoparticle Amplification

4.1 Introduction

Surface plasmon resonance (SPR) biosensors have become an indispensable tool for studying biomolecular interactions in pharmaceutical and biomedical research. Label-free and capable of real-time detection, SPR allows for measurement of analyte concentration and binding kinetics in a fast, convenient, and nondestructive fashion. The Kretschmann configuration SPR is the most widely employed setup, which utilizes a thin (approximately 50 nm) layer of a noble metal (usually gold) deposited on a glass substrate that is attached to a prism¹. By measuring the optical characteristics, including resonance angle, intensity, phase, and polarization, of light reflected from prism, information of biomolecular interactions on the surface is obtained²⁻⁴, including protein conformation⁵, biomarker profiling, aptamer selections⁶ and antibody selections, which have produced high-affinity ligands that specifically recognize protein targets. While highly useful for determining a variety of important kinetic and affinity parameters of biological interactions, one major drawback of the SPR method is the low-throughput nature⁷. This problem has been largely circumvented with the advent of SPR imaging technique. Rather than a scanning-angle or scanning-wavelength measurement commonly employed in SPR spectroscopy, SPR imaging generally measures at a fixed angle where

differences in reflectivity are monitored over time, which provides spatial capabilities of imaging and allows multiplexed detection for high-throughput bioanalysis⁸. By using a CCD camera for signal detection, the images of the chip can be recorded allowing simultaneous analysis of many interactions. SPR imaging has been reported for the bioaffinity detection of proteins and nucleic acids⁹, *Escherichia coli* bacteria¹⁰, and receptor-guest interactions¹¹.

There are multiple new avenues in the SPR imaging research arena, especially when it comes to array fabrication. Microfluidics (Glass/PDMA biomolecular screening chip) offers the distinct advantage of possible individual addressability, which enables multiple chemical environments to be explored in a multiplexed fashion^{12, 13}, but it requires complicated and precise design of flowcells. Contact printing has also been widely used with solid pins, which enable easy deposition of viscous solutions, reproducible and efficient printing, and a simple cleaning procedure¹⁴⁻¹⁶. However, effective passive reagent is necessary after microarray printing to avoid nonspecific adsorption for unarrayed areas¹⁷. SPR imaging analysis is generally displayed through differential sensorgrams¹⁸ or rendered false color images with background subtraction/correction¹⁹⁻²¹. Surface plasmon resonance occurs on the whole surface, giving rise to significant background signals. As the background signal varies in response to surrounding light excitation and solution conditions, the sensitivity and the accuracy of the measurements

in the targeted area can be severely compromised with the fixed background analysis. Some efforts have been made to reduce the background resonance by nonchemical approaches such as the use of a patterned SPR-carrying layer to obtain metal spots or islands separated by uncoated glass^{12, 22, 23}. Here we employ a novel SPR imaging microarray developed earlier in our group²⁴ for protein toxin detection. A layer of 100 nm Ti was deposited on gold sublayer via photolithography to generate Au-well microarray, the spatial variation of the metal thickness restrict the excitation of surface plasmons in the desired patterns and attenuate the evanescent field in the background area.

Recent developments of nanomaterials have remarkably enhanced the detection sensitivity of biosensors. AuNP has been reported to improve the SPR detection sensitivity for biomolecules such as progesterone²⁵, DNA and protein²⁶, but the enhancement is limited. For SPR sensitivity enhancement, packing density of the nanoparticles is important to achieve effective signal amplification. However, rigid structure of large size nanoparticle resists close packing and the vacant areas impaired enhancement effect. To increase the packing density of AuNP on surface, Corn et al. has reported the use of poly(A) polymerase extension reaction coupled with poly(T)-coated AuNP to amplify signals from surface-bound miRNA²⁷. To sufficiently occupy the space among bound AuNPs, polymer growth was generated from AuNP surface to further amplify SPR signal for protein cholera toxin detection²⁸. Small size nanoparticles are

generally less used for signal enhancement in bioassays, because it is difficult to achieve satisfactory packing density using a standard procedure²⁹. Here we develop a new strategy to enhance packing density on surface for SPR signal amplification for toxin detection. For experimental convenience, protein CT was covalently immobilized on a plain gold substrate and Au-well microarray to demonstrate the principle. After surface blocking with BSA and attachment of biotin antiCT and streptavidin, 20nm biotin functionalized AuNP was applied to increase SPR detection signal. These particles also act as building blocks to generate space for subsequent Fe₃O₄NP filling. 5nm streptavidin functionalized Fe₃O₄NP was incubated on surface and attached to biotin functionalized AuNP via biotin-streptavidin interaction, effectively occupied the gap area between AuNPs and further enhance SPR signal. Nonspecific adsorption was significantly reduced due to the high specificity of biotin-streptavidin interaction. The amplification method relies on the accumulation effect of different size conjugated nanoparticles, which sufficiently utilize the space and significantly enhance packing density. Various types of nanoparticles and wide availability of biotinylated antibody would make this method broadly applicable to almost any proteins of interest.

4.2 Experimental

4.2.1 Materials

Streptavidin and (+)-Biotinyl-3,6,9,-trioxaundecanediamine (BA) were obtained from Thermo Scientific (Rockford, IL). N-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), 2-(2'-aminoethoxy) ethanol (AEE), polyoxyethylene (20) sorbitan monolaurate (Tween 20), 16-mercaptohexadecanoic acid (16-MHDA), cholera toxin (CT), 11-mercaptoundecanoic acid (MUA), Bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO). Biotinylated rabbit anti-cholera (Biotinylated antiCT) serum was from ViroStat (Portland, Maine). All proteins solutions were prepared in 20 mM phosphate buffered saline (PBS) (containing 150 mM NaCl, pH 7.4) unless indicated otherwise. Citrate stabilized AuNP solution with the AuNP particle size of about 20 nm was prepared according to the literature³⁰ and stored in brown glass bottles at 4 °C, which is stable for at least 1 month. Poly(acrylic acid) (PAA) wrapped Fe₃O₄NP solution with the Fe₃O₄NP particle size of about 5 nm was prepared according to the literature³¹, which is stable for at least 1 month.

4.2.2 Preparation of AuNP-Hydroxyl-Biotin

AuNP was modified with hydroxyl and biotin groups according to a literature protocol³² with some modification. Citrate stabilized AuNP dispersion with a

concentration of 0.80 nM (determined by measuring absorbance at 520 nm and using an extinction coefficient of $1.25 \times 10^9 \text{ M}^{-1} \text{ cm}^{-1}$) was degassed with nitrogen gas. Tween 20 solution (1.82 mg/mL) was prepared in 10 mM phosphate buffer saline (PBS) at pH 7.0. 16-MHDA solution (0.50 mM) was prepared in degassed ethanol. Equal volumes (400 μL) of AuNP dispersions and Tween 20 solution were gently mixed and allowed to stand for 30 min for physisorption of Tween 20 to the AuNP surface. 400 μL 16-MHDA solution was added and allowed to stand for 3 hr for chemisorption to take place. The final mixture was centrifuged and resuspended in 10 mM PBS (with 1.82 mg/mL Tween 20, pH 7.0) for three times (30 min at 15700 g).

After the final centrifugation step, the 16-MHDA modified AuNPs were reacted with a mixture of freshly prepared 50 mM NHS and 200 mM EDC solution (10 mM PBS, pH 7.0) for 5 min. After washing with 10 mM PBS (with 1.82 mg/mL Tween 20, pH 7.0) (5 min at 15700 g), the remaining NHS ester-alkanethiol modified AuNP were reacted with a freshly prepared aqueous solution of AEE (22 mM) and BA (2.4 mM) for 20 min. After washing with 10 mM PBS (with 1.82 mg/mL Tween 20, pH 7.0) (5 min at 15700 g), the resulted AuNP-Hydroxyl-Biotin was stored at 4 °C.

4.2.3 Preparation of Fe₃O₄NP-Streptavidin

The carboxylate groups of Fe₃O₄NP surface were activated by reacting with a mixture of freshly prepared 100 mM NHS and 400 mM EDC solution (10 mM PBS, pH 7.0) for 5

min. After washing with 10mM PBS, $\text{Fe}_3\text{O}_4\text{NP}$ were incubated with 1.6mg/mL streptavidin solution overnight. After washing with 10 mM PBS, the resulted Fe_3O_4 -Streptavidin was stored at 4°C.

4.2.4 Microarray fabrication

Au-well microarray was fabricated according to our reported protocol²⁴ with modification. As shown in Figure 4.1, a BK7 glass substrate was used for the fabrication with electron-beam evaporation of 2 nm titanium as the adhesion layer and of 48 nm gold as the SPR active layer. A photoresist AZ5214E was then spun coated and patterned by photolithography. A second E-beam evaporation was performed to deposit 100 nm titanium on the patterned substrate. The photoresist was then lifted off using acetone. After rinsing with ethanol and water, the obtained Au-well microarray chips were stored under vacuum before use.

4.2.5 AFM characterization

Atomic force microscopy images were obtained using a Veeco Dimension 5000 atomic force microscope (Santa Barbara, CA) with manufacturer provided software. All images were obtained in tapping mode.

4.2.6 SPR spectroscopy and SPR imaging measurement

A dual channel SPR spectrometer NanoSPR-321 (NanoSPR, Addison, IL) with a GaAs semiconductor laser light source ($\lambda=670$ nm) was used for all SPR spectroscopic

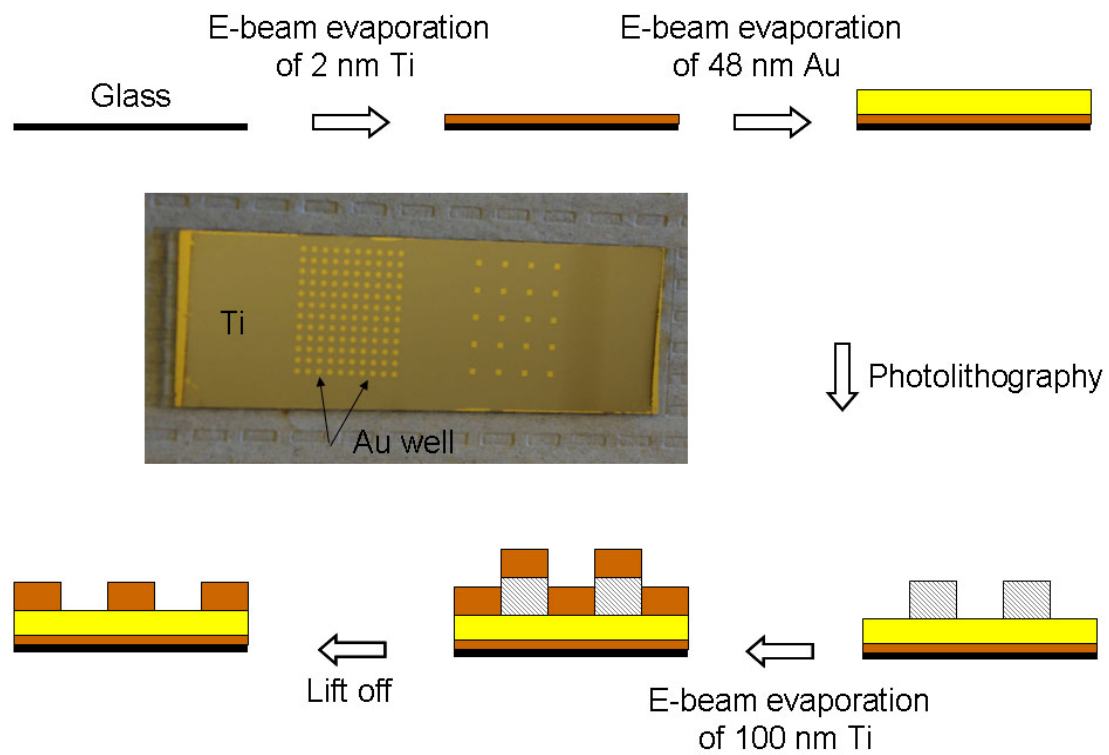


Figure 4.1. Cartoon representation of the fabrication of the gold-well microarray via photolithography.

measurements. The device comes with a high-refractive index prism ($n=1.61$) and 30 μL flow cell. SPR spectroscopy gold chips were fabricated with a 2 nm thick chromium adhesion layer, followed by deposition of a 46 nm thick gold layer via e-beam evaporation onto cleaned BK-7 glass slides. Surface interaction and modification were monitored using the angular scanning mode around the minimum angle.

Au-well microarray chip was mounted on a home made optical stage containing a 300 μL flow cell for SPR Imaging. An equilateral SF2 triangular prism ($n = 1.61$) was then put in contact with the glass substrate with a matching liquid ($n = 1.61$). The optical stage was fixed to a rotating stage that allows the tuning of the incident angle.³³ A red light emitting diode (LED) was used to excite the surface plasmons on the metal surface at a wavelength of 648 nm. The reflected images were captured by a cooled 12-bit CCD camera (Retiga 1300 from QImaging) with a resolution of 1.3 MP (1280 $\times$ 1024 pixels) and 6.7 $\mu\text{m}\times$ 6.7 μm pixel size using p-polarized light. Difference images were then obtained by digitally subtracting one image from another. All the experiments were carried out at room temperature (23 $^{\circ}\text{C}$).

4.2.7 SPR signal amplification with AuNP-Hydroxyl-Biotin and $\text{Fe}_3\text{O}_4\text{NP}$ -Streptavidin

The gold chip for SPR spectroscopy and Au-well microarray chip for SPR Imaging were incubated in 1mM MUA ethanol solution for 18 h to form a self-assembled monolayer

with carboxyl functional group on the surface. After extensive rinsing with copious ethanol and DI water, the chip was dried under a N₂ stream. To activate the carboxyl acid group, EDC (400 mM)/NHS (100 mM) solution was injected into the flow cell and incubated for 30 min followed by 10 min surface rinsing. Varied concentrations of CT were injected subsequently and incubated for 30 min to allow formation of covalent amide linkages, which was followed by a 10 min rinsing to eliminate any residual CT. Passivation of the unused activated carboxyl groups was preformed by incubation with 1mg/mL BSA solution for 30 min. The immunoassay binding was carried out with injection of 0.25 mg/mL biotinylated anti-CT and 30 min incubation. Following this, 0.5 mg/mL streptavidin was injected and incubated for 30 min to prepare the surface for conjugated nanoparticle binding.

The amplification with conjugated nanoparticles attachment was conducted by injection of AuNP-Hydroxyl-Biotin solution and 10 min incubation. After 10 min surface rinsing to remove nonspecific and unstable surface bound AuNP-Hydroxyl-Biotin, Fe₃O₄-Streptavidin was injected subsequently and incubated for 10 min to further enhance SPR signal. The surface was rinsed with water for 10 min to remove unstable surface bound Fe₃O₄-Streptavidin.

4.3 Results and discussion

4.3.1 SPR signal amplification with AuNP-Hydroxyl-Biotin and Fe₃O₄NP-Streptavidin

AuNPs have been widely used to enhance detection signal for SPR biosensors^{34, 35}. However, the amplification effect is not entirely satisfactory due to its rigid structure that limits the packing density on the surface. To overcome this problem, surface enzyme polyadenylation reaction was applied to generate poly(A) tail to increase the binding positions of T₃₀ coated AuNP²⁷, and atom transfer radical polymerization (ATRP) reaction was initiated from AuNP surface to fill the its packing vacancy²⁸. Magnetic nanoparticles have been frequently used in bioseparation and for analyte purification and enrichment³⁶, but there are few reports about their applicability in bioassays due to their extremely small size so that sensitive detection is difficult to achieve using a general procedure^{29, 37}. In this work, 20 nm AuNP-Hydroxyl-Biotin was first applied onto the surface as building blocks to enlarge spatial positions. This is followed by 5 nm Fe₃O₄NP-Streptavidin, which accumulated around AuNP via biotin-streptavidin interaction and sufficiently occupy the gap areas and subsequently increase packing density on surface. The SPR signal enhancement was demonstrated with bacterial cholera toxin (CT). For the sake of experimental simplicity, CT is covalently linked to surface via amide bond to demonstrate the feasibility of conjugate nanoparticle based signal

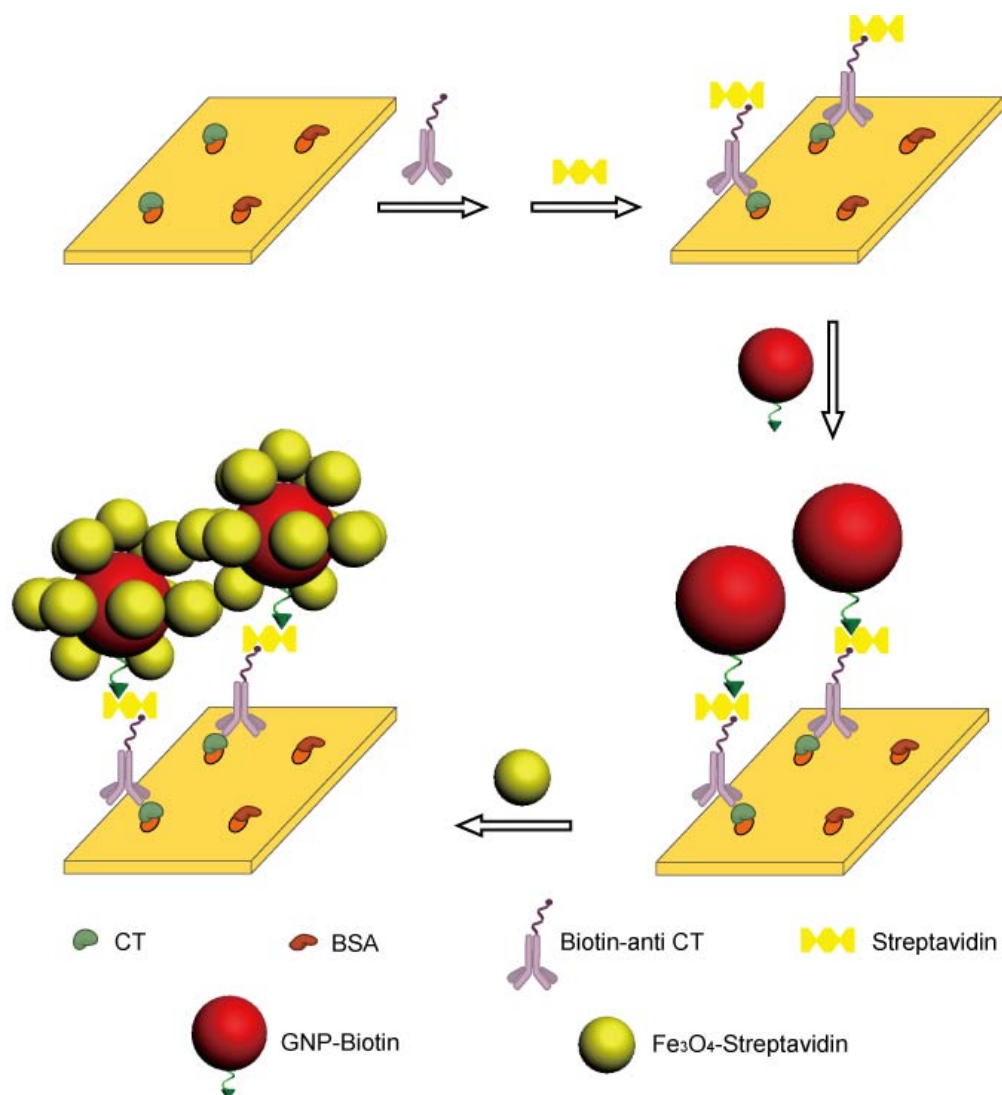


Figure 4.2. Cartoon representation of SPR imaging signal amplification from consecutive different size conjugated nanoparticles binding on the gold well array.

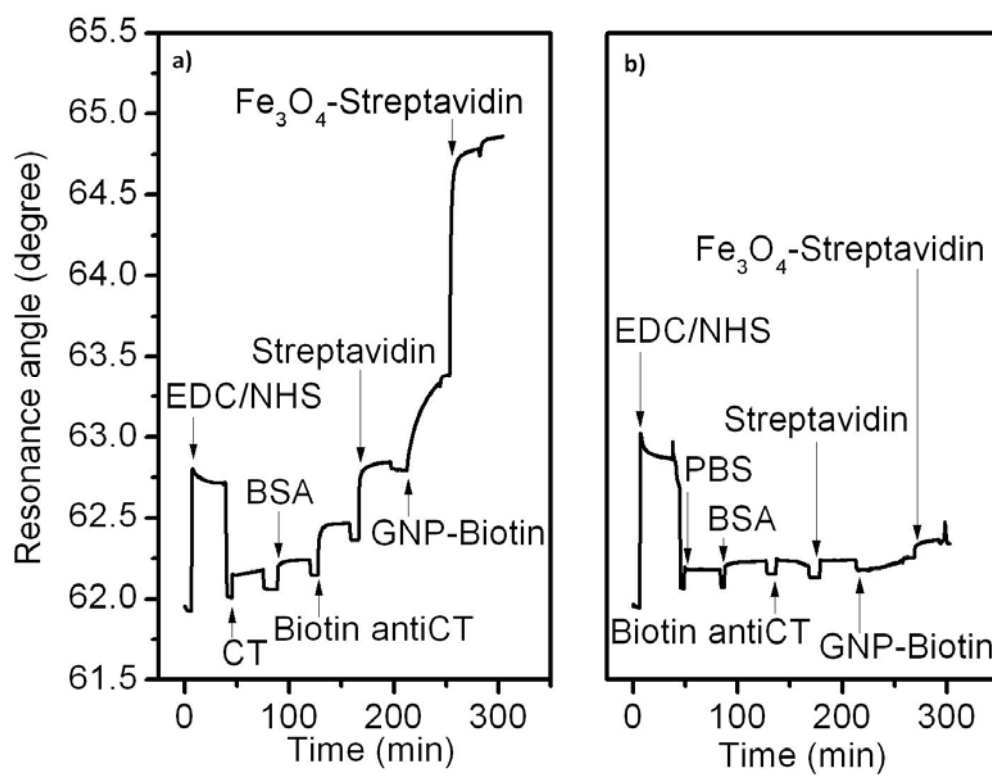


Figure 4.3. SPR sensorgrams for a) immunoassay of 2.51×10^{-13} mol/cm² CT with AuNP-Hydroxyl-Biotin and Fe₃O₄NP-Streptavidin nanoparticle for signal amplification and b) the control channel in the absence of CT.

amplification in SPR (Figure 4.2). To eliminate nonspecific adsorption, the free activated carboxyl acid groups of MUA were blocked by BSA. The surface of attached CT was recognized by injection of biotinylated anti-CT. Then streptavidin was added and specifically bind to the exposed biotin tag. A high concentration of streptavidin was used to ensure effective binding and the captured streptavidins have additional binding sites available for AuNP-Hydroxyl-Biotin in the following step. SPR resonance angle shifted 0.59 degree upon AuNP-Hydroxyl-Biotin binding, and the signal was further increased 1.48 degree for Fe₃O₄NP-Streptavidin attachment (Figure 4.3a). Compared with SPR signal of surface immobilized 0.1 μ M CT (0.05 degree), SPR signal was amplified 40 times by AuNP-Hydroxyl-Biotin and Fe₃O₄NP-Streptavidin attachment. In the control channel, PBS buffer was injected instead of CT under the same condition, followed by surface blocking with BSA and surface incubation with biotinylated antiCT, streptavidin, AuNP-Hydroxyl-Biotin and Fe₃O₄NP-Streptavidin sequentially. There was no measurable SPR signal change for biotin antiCT/streptavidin incubation and little SPR signal increase for AuNP-Hydroxyl-Biotin incubation, demonstrating good specificity of signal enhancement. Unbound Fe₃O₄NP-Streptavidin was not completely removed by surface rinsing due to its small size, which resulted in a 0.10 degree signal (Figure 4.3b). However, this signal increase is negligible compared with substantial specific binding signal (1.48 degree Figure 4.3a).

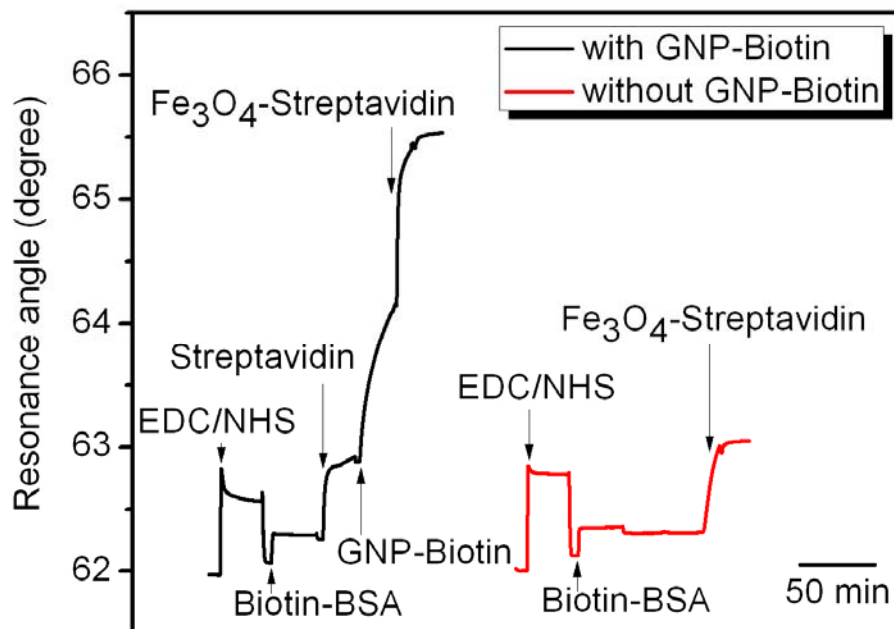


Figure 4.4. SPR sensorgrams for $\text{Fe}_3\text{O}_4\text{NP-Streptavidin}$ nanoparticle binding on covalently immobilized Biotin-BSA in the presence (black) and absence (red) of $\text{AuNP-Hydroxyl-Biotin}$ nanoparticle.

To demonstrate the contribution of AuNP-Hydroxyl-Biotin to Fe₃O₄NP-Streptavidin accumulation, Biotin-BSA was immobilized on surface and the signal from direct binding of Fe₃O₄NP-Streptavidin to Biotin-BSA was compared to the binding signal of Fe₃O₄NP-Streptavidin in the presence of AuNP-Hydroxyl-Biotin (Figure 4.4). With streptavidin as the connection bridge and AuNP-Hydroxyl-Biotin as building blocks, Fe₃O₄NP-Streptavidin effectively occupied the space around AuNP and generated 1.39 degree of SPR signal increase. In the absence of AuNP, biotin-BSA only provided limited space for Fe₃O₄NP-Streptavidin attachment, resulting in a 0.74 degree of SPR signal increase. AuNP-Hydroxyl-Biotin attached on surface not only enhanced SPR signal to a certain extent, but also increased spatial positions for Fe₃O₄NP-Streptavidin accumulation. As it demonstrated in Figure 4.4, the increased spatial positions amplified binding signal of Fe₃O₄NP-Streptavidin about two times.

4.3.2 Morphological characterization of Fe₃O₄NP-Streptavidin and AuNP-Hydroxyl-Biotin nanoparticle conjugates

The accumulation of Fe₃O₄NP-Streptavidin around AuNP-Biotin was characterized by AFM which is capable of providing useful topographic information for surface immobilized nanoparticles. Figure 4.5 shows the surface feature of adsorbed Fe₃O₄-Streptavidin and AuNP-Hydroxyl-Biotin with 0.1 μ M CT immobilization. Unlike individual dots isolated on surface for sole AuNP binding³⁸, block conjugates

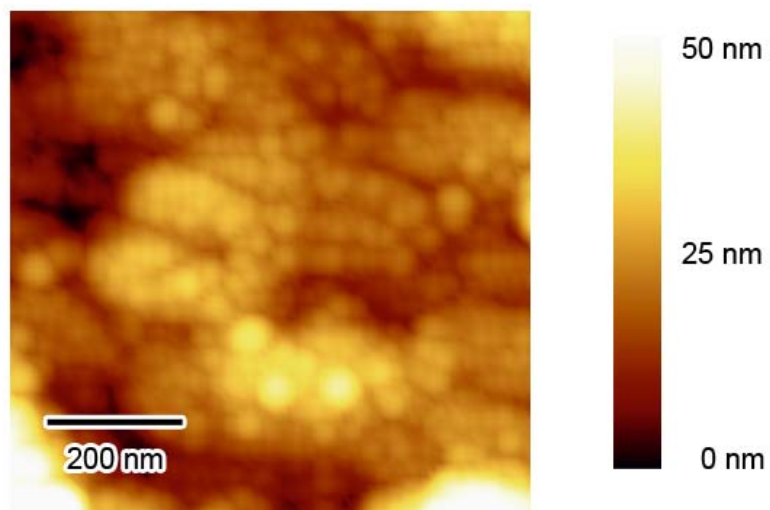


Figure 4.5. AFM image of Fe₃O₄NP-Streptavidin and AuNP-Hydroxyl-Biotin conjugate nanoparticles binding on sensorchip in the presence of $2.51 \times 10^{-13} \text{ mol/cm}^2$ CT.

were observed densely packed on surface which consists of small particles, resulting island with thickness above 30 nm and average feature size over 100 nm. The average size of individual small particle is about 10 nm, appropriately equals to the sum of $\text{Fe}_3\text{O}_4\text{NP}$ (5 nm)³¹ and streptavidin (3-5 nm)³⁹. This confirmed Fe_3O_4 -Streptavidin accumulated around AuNP-Hydroxyl-Biotin, fully occupied the special area and significantly enhance nanoparticle packing density on surface.

4.3.3 Highly sensitive detection of toxin.

CT is responsive for the serious effects of cholera infection, one of the most severe illnesses in developing countries. The lethal dose for cholera toxin in human is relatively low ($\text{LD}_{50} = 250 \mu\text{g kg}^{-1}$), therefore there is a clinical need for accurate detection of CT with high sensitivity⁴⁰. In recent years, ultrasensitive detection of CT has drawn considerable interest⁴¹. As a model protein to demonstrate SPR signal enhancement, CT was covalently immobilized on surface to simplify the experiment design. Figure 4.6 shows a plot of the SPR signal increase as a function of CT surface coverage after immuno-recognition and conjugate nanoparticle binding, as illustrated in Figure 4.1. The surface coverage of CT was calculated based on our formerly reported method⁴². The conjugated nanoparticle amplified signal appears to show two segments of linear correlation to CT concentration. When surface immobilized CT amount is between 2.51×10^{-13} to $7.94 \times 10^{-13} \text{ mol/cm}^2$, the large protein amount generated substantial signal

increase based on conjugated nanoparticle binding, resulted in a much steeper calibration curve. This type of segmental calibration was also reported by our formerly reported detection method for CT detection²⁸ and other group for IgG fluorescence detection⁴³. Another linear range is from 6.31×10^{-16} mol/cm² to 2.51×10^{-13} mol/cm² ($R^2=0.9322$), which is more significant to ultrasensitive detection. Even at 6.31×10^{-16} mol/cm² (the concentration of CT used in immobilization is 4.52 pM), the immobilized CT generated discernible SPR signal change after conjugated nanoparticle binding, which is much lower than most previously reported CT detection methods including SPR signal amplification with ATRP polymer growth⁴², colorimetric method⁴⁴, fluorescently labeled microarray⁴⁵, electrochemical biosensors relied on bilayer lipid membrane supported on glass fiber⁴⁶ or with a microfluidic technique⁴⁷. With the use of the 3S/N principle, the detection limit was determined to be 5.01×10^{-16} mol/cm². Taking the flow cell area into calculation, the absolute quantity of detection limit is 0.25 fmol, which is comparable to high sensitive enzyme-catalyzed enhanced chemiluminescence reaction⁴⁸.

4.3.4 SPR imaging characterization of Au-well array and signal amplification measurement.

The SPR imaging experiments were carried out using a home-built instrument arranged in the Kretschmann configuration³³. The reflectance from the array was imaged with a CCD camera and the change in reflectance was recorded in real-time. Contact

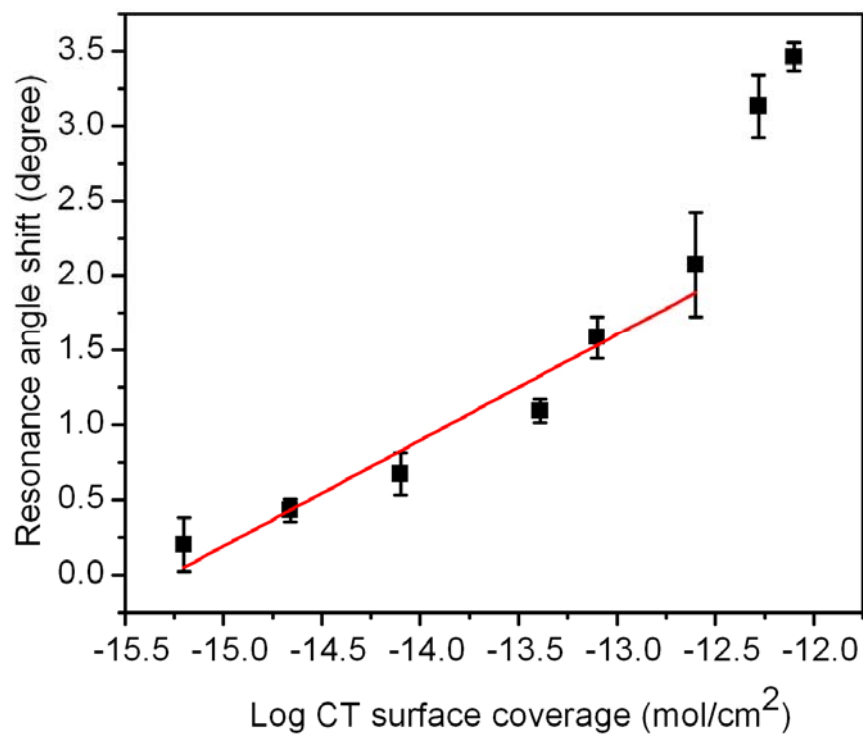


Figure 4.6. SPR response as a function of CT surface coverage after AuNP-Hydroxyl-Biotin and Fe₃O₄NP-Streptavidin conjugate nanoparticle amplification with streptavidin bridge.

printing^{33, 49}, microfluidic cross-patterning with two PDMS chips⁵⁰ and automatic arrayer technique⁵¹ have been used to generate protein array on flat gold substrate for SPR imaging. However, effective blocking method is required to deactivate the unbound area and the image quality was restricted by background resonance interference and nonspecific adsorption. Recently, we developed a microarray of 800 μm diameter gold well surrounded by raised titanium platform to attenuate the evanescent field in the background area²⁴. As it demonstrated in Figure 4.7a, surface plasmons were only excited in Au well areas, which shows black dots. S-polarized light does not generate any SPs, but can offer image background for comparison. With the immobilization of 1.29×10^{-12} mol/cm² CT, the following immunoassay binding of biotin AntiCT, streptavidin and conjugated nanoparticle attachment significantly increased surface refractive index, made the reflected images turn to visibly bright (Figure 4.7b). To deduct boundary background and make the binding signal stand out, difference image was obtained using Figure 4.7a and 4.7b. The high reflection intensity from conjugate nanoparticle amplification clearly demonstrates CT immobilization (Figure 4.7c). In comparison, a difference SPR image was captured for 1.29×10^{-12} mol/cm² CT before antibody binding and conjugate nanoparticle signal enhancement: the CT immobilization signal was barely observable (Figure 4.7d). The corresponding 3D (Figure 4.7e) and 2D (Figure 4.7f) profiles were generated for the array elements from Figure 4.7c, demonstrating the high reproducibility

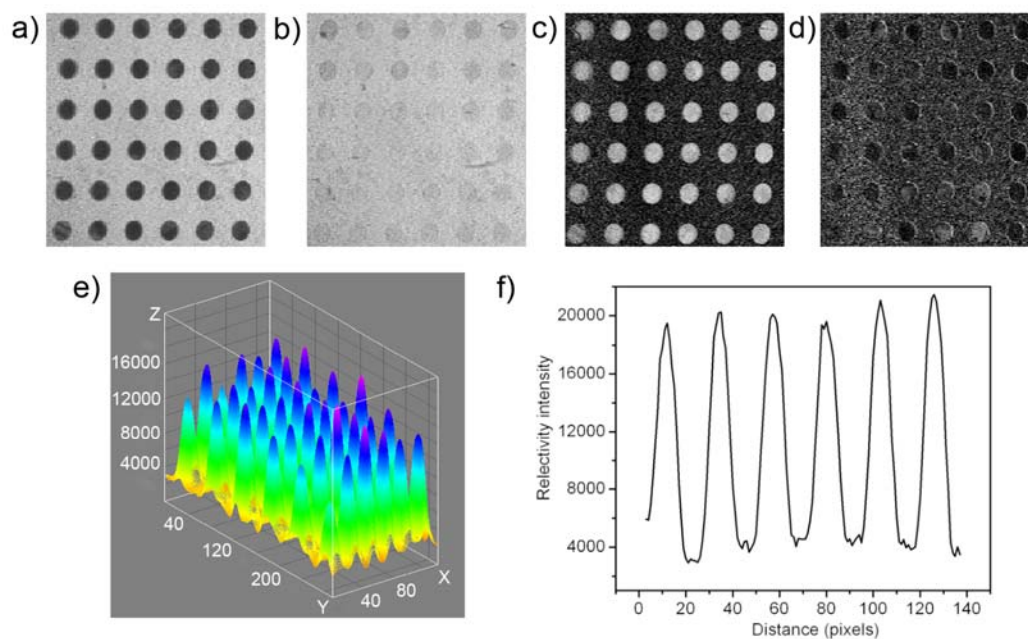


Figure 4.7. Raw SPR images of gold well microarray a) before CT immobilization and b) $1.29 \times 10^{-12} \text{ mol/cm}^2$ CT after nanoparticles amplification. c) Difference SPR images of $1.29 \times 10^{-12} \text{ mol/cm}^2$ CT after nanoparticle amplification by subtracting a) from b), and d) before amplification. The corresponding e) 3D and f) 2D intensity profile for the array elements.

of the array. SPR imaging quantitative analysis of different concentration CT were also performed and the result was shown in Figure 4.8. Each image was a 2 x 5 array for CT concentration ranged from 3.16×10^{-15} to 1.29×10^{-12} mol/cm², and the difference images were taken by subtracting background image which was taken before CT immobilization. Reflection intensities after conjugated nanoparticle signal enhancement are also listed. For comparison, a control array was fabricated in the absence of CT (Figure 4.8), the surface was blocked by BSA and subjected to the same experimental conditions for immunoassay binding and conjugate nanoparticle signal amplification. There was only a small signal (RI = 803 ± 52.3 au) obtained, suggesting the high specificity of conjugated nanoparticle binding signal enhancement. With the CT concentration increasing, the reflection intensity difference became pronounced. The highest signal amplification is obtained with 1.29×10^{-12} mol/cm² CT immobilization (RI = 16540 ± 216.59), while 3.16×10^{-15} mol/cm² CT generated signal increase of 1667 ± 107.2 . The reflection intensity was plotted against CT surface coverage, demonstrating a linear relationship from 3.16×10^{-15} to 1.29×10^{-12} mol/cm². Using 3 S/N cutoff, the detection limit was determined to be 2.0×10^{-15} mol/cm². Taking the individual Au-well microarray area into calculation, the absolute quantity of detection limit is 3.2 amol, which is much lower than ultrasensitive electrochemistry detection⁴⁰ and fluorescence detection with signal amplification by fluorescent nanoparticles⁵², and 10^5 times more sensitive than direct CT

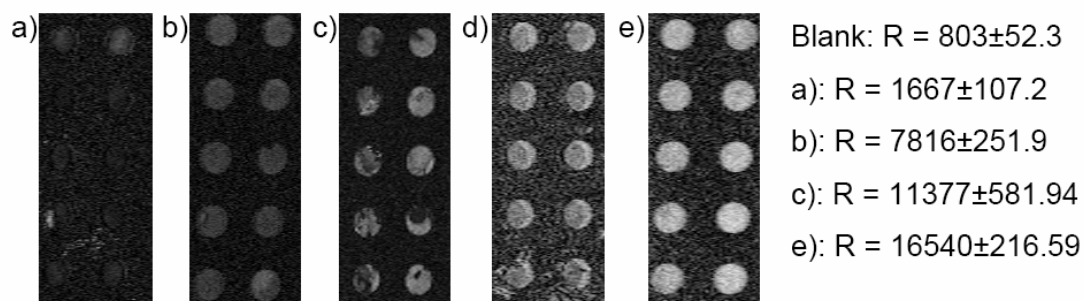


Figure 4.8. SPR images of 2x5 arrayed gold wells with a) control, b) $3.16 \times 10^{-15} \text{ mol/cm}^2$, c) $7.59 \times 10^{-15} \text{ mol/cm}^2$, d) $1.00 \times 10^{-13} \text{ mol/cm}^2$ and e) $1.29 \times 10^{-12} \text{ mol/cm}^2$ after Fe_3O_4 -Streptavidin and AuNP-Biotin conjugate nanoparticle amplification.

detection on Au-well microarray via SPR imaging.⁵³ By choosing appropriate biotinylated antibody, this method can be applied to high-throughput detection of many proteins.

4.4 Conclusion

We report here a novel SPR signal amplification strategy for highly sensitive detection of proteins by stacking different size of nanoparticles. Immunoassay binding of biotinylated antibody to the target protein and subsequent attachment of streptavidin is attained on gold substrate followed by specific SPR signal amplification from two different size of conjugated nanoparticles. Two consecutive nanoparticle binding of 20nm AuNP-Hydroxyl-Biotin and 5nm Fe₃O₄NP-Streptavidin greatly increase packing density on surface thus enhance SPR signal substantially. The immobilized CT has been detected in the range of 6.31×10^{-16} to 2.51×10^{-13} mol/cm² and a detection limit as low as 6.31×10^{-16} mol/cm² (0.25 fmol) has been obtained.

An Au-well microarray of 800 μ m diameter was used for SPR Imaging detection. The evanescent field is attenuated in the background area and SPR resonance is restricted in gold wells, therefore avoid background resonance interference. CT detection from Au-well microarray demonstrated a linear relationship from 3.16×10^{-15} to 1.29×10^{-12}

mol/cm² with a detection limit of 2.0×10^{-15} mol/cm², the absolute quantity of detection limit is 3.2 amol considering the small size of Au-well microarray. This method can be applied to high throughput detection of various proteins.

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Chapter 5 Protein and Small Molecule Recognition Properties of Deep Cavitands in a Supported Lipid Membrane Determined by Calcination-Enhanced SPR Spectroscopy

5.1 Introduction

The surfaces of mammalian cells are decorated with a wide variety of membrane-bound receptor molecules. These receptors act as sensors for, and transporters of small molecules in the extracellular environment¹. For example, acetylcholinesterase is a membrane-bound protein that binds the target acetylcholine via non-covalent cation- π interactions with active site aromatic residues². These natural receptors have inspired the discovery of new methods to transport polar drug candidates across hydrophobic membrane bilayers, a process of great importance to medicinal chemistry³. Small molecules have been exploited to induce endocytosis⁴ or to hydrophobically shield the drug candidate as it is transported through the membrane⁵. Many examples of targeted drug delivery use natural receptors such as glycolipids⁶ or polypeptides⁴ to recognize the target, or study the transport process alone by covalently linking the drug target to a suitable vector⁵. In these cases, the artificial membrane receptors typically consist of the covalent attachment of the recognition motif to a lipid or steroid derivative that is incorporated in a synthetic membrane. The recognition motif is displayed above the membrane surface in order to bind to the target.

An alternate method of substrate recognition is the incorporation of a defined binding pocket *inside* the membrane bilayer. This strategy is employed by membrane-penetrating proteins⁷ and transmembrane pore-forming peptides⁸ – the host is incorporated in the

membrane itself, and displays a cavity that allows polar substrates to be shielded from the lipophilic membrane⁹. This is mainly exploited for substrate transport *through* the membrane, but a synthetic host that displayed this type of binding motif would show a far greater range of application than receptors created *via* the covalent derivatization of steroids and lipids. To achieve this, we require a synthetic cavity that can be incorporated into a membrane bilayer while still retaining selective host properties. Most water-soluble synthetic host molecules take advantage of the hydrophobic effect to recognize their desired targets¹⁰, which poses a problem for application in natural systems. Hydrophobic substrates suffer from poor water-solubility and non-specific localization in the lipophilic membrane bilayers present in cells. In addition, competitive binding of hydrophobic lipids themselves inside the host will reduce the affinity for the target substrate. Because of these factors, the use of cavity-based synthetic receptors in natural systems is underexplored.

Deep cavitands¹¹ are well-known as protein mimics, in that they provide a cavity that can selectively recognize molecules of the correct shape if they also contain a thin layer of positive charge at their surface¹². Excellent selectivity for substituted trimethylammonium salts is possible in both water¹³ and organic solvents¹⁴. Recognition of hydrocarbons¹⁵ and steroids¹⁶ *via* the hydrophobic effect has also been reported. As well as binding hydrophobic species, molecules such as Rebek's tetracarboxylate cavitand **1** (Figure 5.1) have shown the ability to be incorporated in lipid micelles¹⁷. The host:guest properties of these small micellar aggregates were studied by 1D NMR and diffusion NMR techniques, indicating that the molecules were able to bind suitable guests

in the presence of lipids above the critical micelle concentration, albeit with reduced affinities with respect to those in pure water. Analysis of the composition of the micelles was not performed, nor were studies carried out on larger aggregates such as vesicles or membrane bilayers. In order to apply this proof of principle to more biorelevant settings, analysis of the host properties in bilayers and vesicles is required. NMR analysis of the micellar properties provides some information, but ^1H NMR spectroscopy is poorly suited for analysis of membrane bilayers due to substantial signal broadening observed. A different sensing technique is required.

Supported lipid bilayers are an excellent mimic of natural membranes, as they maintain similar fluidity properties¹⁸ and substrate mobility. These membrane mimics have been successfully used for the study of a variety of biological phenomena¹⁹. The predominant technique for characterizing activities on the membrane has been fluorescence microscopy, while in recent years label-free methods such as surface plasmon resonance have gained considerable application due to simple experimental procedure and real-time measurement capability²⁰. Proper functionalization of the gold substrates by hydrophilic polymers in SPR is required in order to fabricate quality membrane mimics. Recently an improved approach using a calcinated nanoglassified gold surface has come forward and allowed direct assembly of the supported membranes at the sensing interface²¹, allowing the real-time detection of binding events in a supported membrane bilayer. The binding properties of proteins such as lectins²² and bacterial toxins²³ have been extensively assessed with the calcinated gold chips.

Synthetic host molecules, such as calixarenes, shallow cavitands and cyclodextrins, have been directly attached to surfaces for sensing applications²⁴. They have been tested for detection of a range of small molecules including gas vapors, polycyclic aromatic hydrocarbons via surface-enhanced Raman spectroscopy (SERS)²⁵, and adrenaline/catecholamine via electrochemistry²⁶. The results show satisfactory sensitivity, however detection of small molecules by SPR can be challenging to implement as low molecular weight compounds are insufficiently large to generate a measurable refractive index change. SPR sensing of small molecule interactions in a supported bilayer poses an even greater challenge, as the recognition event is displaced from the surface. In addition, the binding constants obtainable by synthetic receptors are generally on the order of millimolar (with some notable exceptions²⁷), rather than the micro- and nanomolar binding affinities displayed by proteins. Binding properties of lectins that show millimolar binding affinities are detectable²⁸, but any receptor incorporated in the membrane must display at least millimolar affinity for substrate in order for accurate analysis to be possible.

In this work, the host-guest interactions of small molecule with cavitand incorporated supported bilayer lipid membrane are investigated by SPR spectroscopy. The use of calcinated surface offers an enhanced mode of SPR detection, allowing study of the interactions of cavitands such as **1** with a series of choline derivatives guests in a near-native cell membrane mimic. The binding events are exploited by SPR and fluorescence microscopy to understand the interactions between polar guests and the water-soluble

host (cavitand) in biomimetic media, while the employment of biotin-tagged guests aims to further explore the protein binding properties at the membrane surface via a host:guest handle.

5.2 Experimental

5.2.1 Instruments

^1H spectra were recorded on a Varian Inova 400 spectrometer. Proton (^1H) chemical shifts are reported in parts per million (δ) with respect to tetramethylsilane (TMS, $\delta=0$), and referenced internally with respect to the protio solvent impurity. Deuterated NMR solvents were obtained from Cambridge Isotope Laboratories, Inc., Andover, MA, and used without further purification. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine was purchased from Avanti Polar Lipids. All other materials were obtained from Aldrich Chemical Company, St. Louis, MO and were used as received. Solvents were dried through a commercial solvent purification system (SG Water, Inc.).

5.2.2 Synthesis of cavitands and guests

Cavitands **1-3** were synthesized according to literature procedures¹⁰, guests 5-8 were synthesized according to the following procedure.

2-Thioureidonaphthalene-ethyltrimethylammonium chloride 5

2-Naphthyl isothiocyanate (19 mg, 0.10 mmol) was added to a solution of 2-aminoethyl- trimethylammonium chloride (18 mg, 0.10 mmol) and Et_3N (10 μL) in DMSO (0.6 mL). After stirring for 1 hr at 70 $^\circ\text{C}$, the solution was added to acetone (5 mL) and hexane (5 mL). The oily residue was separated by centrifugation, then added to 10%

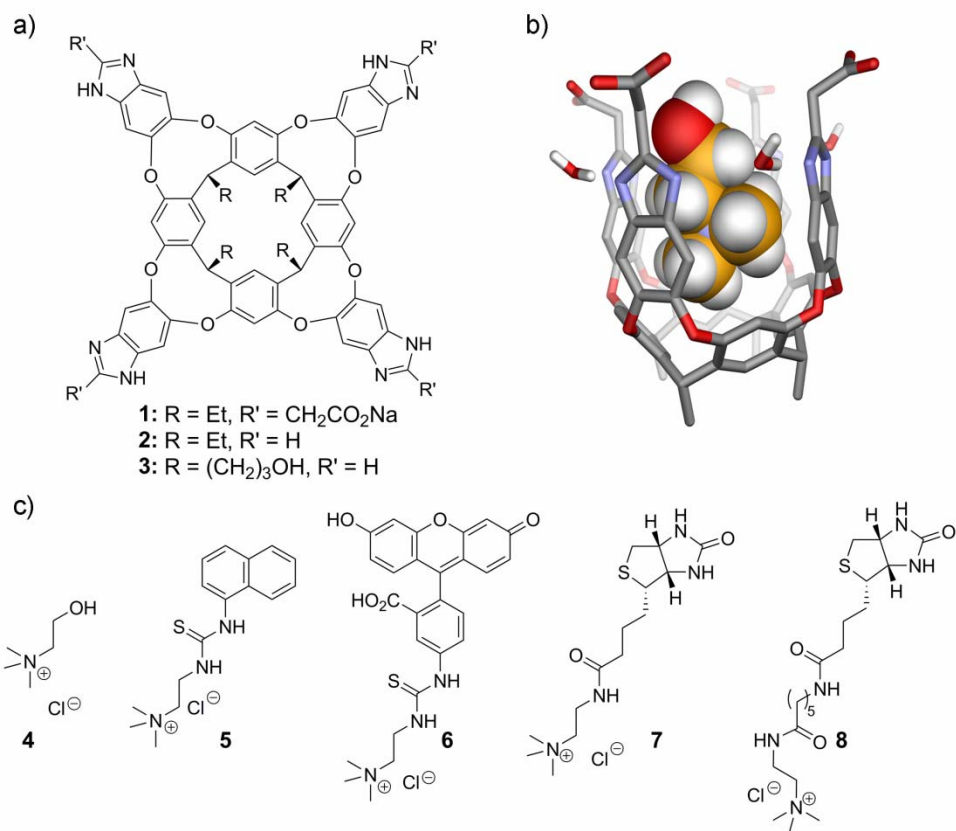


Figure 5.1. a) Cavitands **1-3**; b) minimized conformation of **1** (SPARTAN, AM1 forcefield) with one bound choline molecule (**4**) in the cavity; c) the guests used in this study.

aqueous NaOH solution (1.5 mL) and washed with ethyl acetate (5 mL $\times$ 2). After neutralizing with 10% HCl solution, the solvent was removed *in vacuo* to furnish product **5** (15 mg, 46%). ^1H NMR (400 MHz, DMSO- d_6) δ 11.93 (s, 1H), 10.40 (s, 1H), 8.20 (s, 2H), 7.80 (dd, J = 8.6 Hz, 17.7, 7H), 7.69 (dd, J = 2.1 Hz, 8.8, 2H), 7.42 (dt, J = 7.5 Hz, 15.9 Hz, 4H), 3.56 (t, J = 6.3 Hz, 4H), 3.31 (t, J = 6.9 Hz, 4H), 3.17 (s, 9H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 182.27, 139.00, 134.09, 131.32, 128.47, 128.17, 127.11, 125.66, 124.32, 119.38, 105.16, 64.80, 53.68, 38.52. HRMS (ESI) m/z : calcd for $\text{C}_{16}\text{H}_{22}\text{N}_3\text{S}$ (M^+) 288.1529; found 288.1537.

2-(Thioureidofluorescein)-ethyltrimethylammonium chloride 6

Fluorescein isothiocyanate (39 mg, 0.10 mmol) was added to a solution of 2-aminoethyl- trimethylammonium chloride (17 mg, 0.10 mmol) and K_2CO_3 (28 mg, 0.20 mmol) in water (1.5 mL). After stirring for 18 hr at room temperature, the solution was filtered and the filtrand was added to acetone (5 mL). The precipitate was separated by centrifugation and washed with acetone (3 mL) twice providing the desired compound **6** as a red solid (18 mg, 34%). ^1H NMR (400 MHz, D_2O) δ 7.70 (d, J = 2.1 Hz, 1H), 7.50 (dd, J = 2.2 Hz, 8.2 Hz, 1H), 7.21 (m, 3H), 6.68 – 6.60 (m, 4H), 4.13 (t, J = 6.6 Hz, 2H), 3.63 (t, J = 6.8 Hz, 2H), 3.25 (s, 10H); ^{13}C NMR (100 MHz, D_2O) δ 180.79, 174.09, 157.75, 148.99, 143.98, 141.19, 131.49, 130.03, 126.16, 124.86, 123.02, 112.52, 103.77, 63.91, 53.54, 38.73; HRMS (ESI) m/z : calcd for $\text{C}_{26}\text{H}_{26}\text{N}_3\text{O}_5\text{S}$ (M^+) 492.1588; found 492.1587.

2-Biotinamidyl-ethyltrimethylammonium chloride **7**

Isobutyl chloroformate (16 μ L) was added to a solution of biotin (25 mg) in DMF (0.6 mL) containing tri-N-butylamine (32 μ L). After 10 min at room temperature, the mixture was added to a solution of 2-aminoethyl-trimethylammonium chloride (18 mg, 0.10 mmol) in DMF/water (1:1). After stirring at room temperature overnight, added to 10% NaOH solution (1.5 mL) and washed with ethyl acetate (5 mL $\times$ 2). After neutralizing with 10% HCl solution, the solvent was removed *in vacuo* to furnish product **7** (18 mg, 49%).

^1H NMR (400 MHz, D_2O) δ 4.66 (dd, J = 4.7 Hz, 7.8, 1H), 4.47 (dd, J = 4.5 Hz, 7.9, 1H), 3.74 (t, J = 6.3 Hz, 2H), 3.54 (t, J = 6.7 Hz, 2H), 3.44 – 3.34 (m, 1H), 3.23 (s, 9H), 3.05 (q, J = 5.1 Hz 1H), 2.83 (d, J = 13.1 Hz, 1H), 2.35 (t, J = 7.3 Hz, 2H), 1.84 – 1.56 (m, 4H), 1.52 – 1.38 (m, 2H); ^{13}C NMR (100 MHz, D_2O) δ 177.54, 165.52, 64.34, 62.26, 60.42, 55.52, 53.50, 39.84, 35.41, 33.59, 28.07, 27.81, 24.97. HRMS (ESI) m/z : calcd for $\text{C}_{15}\text{H}_{29}\text{N}_4\text{O}_2\text{S}$ (M^+) 329.2006; found 329.2007.

2-Biotinamidyl-caproylethyltrimethylammonium chloride **8**

Isobutyl chloroformate (9 μ L) was added to a solution of biotinylaminohexanoic acid²⁹ (18 mg) in DMF (0.6 mL) containing tri-N-butylamine (30 μ L). After 10min at room temperature, the mixture was added to a solution of 2-aminoethyl-trimethylammonium chloride (9 mg, 0.05 mmol) in DMF/water (1:1). After stirring at room temperature overnight, added to 10% NaOH solution (1.5 mL) and washed with ethyl acetate (5 mL $\times$ 2). After neutralizing with 10% HCl solution, the solvent was removed *in vacuo* to furnish product **8** (20 mg, 83%). ^1H NMR (400 MHz, D_2O) δ 4.64 (dd, J = 5.0, 8.0, 1H),

4.46 (dd, $J = 4.5, 7.9$, 1H), 3.72 (t, $J = 6.7$, 2H), 3.51 (t, $J = 6.7$, 2H), 3.44 – 3.33 (m, 1H), 3.21 (s, 9H), 3.03 (q, $J = 5.0$ Hz, 1H), 2.82 (d, $J = 13.1$ Hz, 1H), 2.35 – 2.24 (m, 4H), 1.82 – 1.50 (m, 8H), 1.48 – 1.40 (m, 2H), 1.39 – 1.30 (m, 2H). ^{13}C NMR (100 MHz, D_2O) δ 177.52, 176.79, 165.50, 64.32, 62.24, 60.41, 55.52, 53.50, 39.87, 39.19, 35.63, 33.60, 28.16, 27.98, 27.83, 25.76, 25.35, 24.88, 23.37. HRMS (ESI) m/z : calcd for $\text{C}_{21}\text{H}_{40}\text{N}_5\text{O}_3\text{S}$ (M^+) 442.2846; found 442.2845.

5.2.3 Calcinated chip preparation

Gold substrates were fabricated with a 2 nm thick chromium adhesion layer, followed by deposition of a 46 nm thick gold layer *via* e-beam evaporation onto cleaned glass slides. The nanoglassified layers were constructed on the surface based on a previous layer-by-layer protocol³⁰. Clean gold substrates were immersed in 10 mM 3-mercaptopropionic acid (MPA) ethanol solution overnight to form a self-assembled monolayer. After extensive rinsing with ethanol and nanopure water and drying with nitrogen gas, modified gold substrates were alternated dipped into sodium silicate solution (22 mg/mL, adjusted to pH 9.5) and poly(allylamine hydrochloride) solution (1 mg/mL, adjusted to pH 8.0) for 1 min to form a layer by layer assembly structure, with sufficient nanopure water rinsing between layers. This dipping process was repeated eight times to build up a multilayered chip, followed by calcination in a furnace by heating to 450 °C at a rate of 17 °C per min and subsequent cooling to room temperature 4 hours later.

5.2.4 Vesicle preparation

PC lipid stock solution was transferred to a small vial and the organic solvent was purged from the vial with N₂ to form a dry lipid film on the vial wall, which was then rehydrated with 20 mM phosphate buffered saline (150 mM NaCl, pH 7.40) to a lipid concentration of 1 mg/mL. The resuspended lipids were probe sonicated for 20 min, followed by centrifugation at 8000 rpm for 6 min to remove any titanium particles released from the probe tip. The supernatant was then extruded with 11 passes through a polycarbonate membrane of pore size 100 nm to ensure formation of small unilamellar vesicles. The solution was then incubated at 4 °C for at least 1 h before use.

5.2.5 Fabrication of cavitand **1 receptor layer and guest binding measurement**

The fabrication of cavitand **1** - membrane complex and subsequent guest binding was monitored through surface plasmon resonance (SPR) spectrometry and fluorescence microscopy. The shift of SPR minimum angle characterized surface thickness and surface refractive index change, demonstrating adsorption or binding on the surface. The calcinated gold substrate was first rinsed with ethanol and nanopure water and after drying under a gentle stream of N₂ gas, then was clamped down by a flow cell on a high-refractive index prism for SPR measurement. 1 mg/mL PC vesicles in 20mM phosphate buffered saline (150 mM NaCl, pH 7.40) were injected through a flow-injection system, and incubated for 1 h to allow vesicle fusion on the hydrophilic calcinated gold surface, forming a smooth bilayer membrane. After 10 min rinsing to remove excess vesicle from the surface, 2 mg/mL cavitand **1** in 10% DMSO solution was subsequently injected and

incubated for 20 min. The surface was extensively rinsed with nanopure water, followed by incubation with 2 mM aqueous solution of guest **4-8** for varied times. Control experiments were performed under identical conditions in the absence of cavitand **1**.

For fluorescein guest **6**, fluorescence microscopy experiments were also performed on the same chip setup. After sufficient rinsing with nanopure water, fluorescence microscopy was carried out on a Zeiss LSM 510 confocal laser scanning microscope with 488 nm argon laser excitation and a CCD camera. For comparison, an aqueous solution of guest **6** was injected onto the PC membrane surface as before, in the absence of cavitand **1**.

5.2.6 Surface coverage calculations

The surface coverage of adsorbates on a membrane bilayer can be estimated via Jung's formula (eq 1) when the membrane between adsorbate and metal surface is very thin compared to plasmon decay length³¹. Cavitand **1** surface coverage (in units of molecules/cm²) can be estimated by the SPR signal increase:

$$\theta = N \times \frac{I_d}{2} \times \frac{R}{m \times (\eta_a - \eta_s)} \quad (5-1)$$

where N is the bulk number density of the adsorbate; I_d is roughly estimated as 0.37 of the light wavelength (670 nm here); R is SPR response (in the unit of degrees) via binding; m is a instrument constant (determined experimentally by calibrating the measured sensor response to changes in refractive index); η_a is the refractive index of

adsorbed molecule and η_s is the refractive index of bulk solution (1.33 for water). I_d is much larger than the thickness of bilayer lipid membrane (~5 nm), allowing correct application of equation (5-1)³¹.

To calculate the surface coverage of cavitand **1**, the bulk refractive index of cavitand **1** was estimated as 1.6013 and bulk density was estimated as 1.1254 g/cm³³². From SPR signal increase upon cavitand **1** binding (0.20 ± 0.02 degree), θ was determined to be $8.02 \pm 0.93 \times 10^{13}$ molecules/cm². NeutrAvidin binding to the **1•7** complex caused a signal increase of 0.25 ± 0.05 degree. Crystalline protein refractive index (1.60) was used as bulk refractive index,²⁸ and 1.43 g/cm³ as bulk density³³, thus θ of NeutrAvidin was determined to be $2.94 \pm 0.62 \times 10^{12}$ molecules/cm².

5.2.7 Kinetic analysis

Saturation binding mode (eq 2)²⁰ was applied here to determine the equilibrium dissociation constant (K_D) value for the interaction between cavitand **1** and guest **6**. Increasing concentrations of guest **6** (0.5 mM to 5 mM) were injected over the cavitand **1**:membrane complex, and the minimum angle shift was recorded:

$$AB_{eq} = AB_{max} \times \frac{1}{1 + \frac{K_D}{[A]}} \quad (5-2)$$

where AB_{eq} is the average of response signal at equilibrium and AB_{max} is the maximum response that can be obtained for guest **6** binding and $[A]$ is the concentration of guest **6**

injection. $AB_{\max}/AB_{\text{eq}}$ was plotted against $1/[A]$, and the slope is equal to K_D value (3.54 ± 0.92 mM). K_A , the equilibrium association constant ($282 \pm 73 \text{ M}^{-1}$), can be determined by the reciprocal value of K_D . This process was used to determine K_D/K_A for guests **5**, **7**, **8** and NeutrAvidin.

In transient SPR response mode³⁴, the initial rate kinetic method was employed to extract association rate constant (k_{assoc}), dissociation rate constant (k_{diss}), and K_D value for the interaction between cavitand **1** and guest **6**. At $t = 0$, the equation for initial rate analysis is

$$\frac{d_{AB}}{d_t} = AB_{\max} \times [A] \times k_{\text{assoc}} \quad (5-3)$$

By plotting the initial rate against $[A]$, k_{assoc} was calculated as $289.76 \text{ M}^{-1}\text{min}^{-1}$. k_{diss} can be determined from the dissociation curve of SPR sensorgram:

$$AB_t = (AB_0 - AB_{\infty}) \times e^{-k_{\text{diss}}t} + AB_{\infty} \quad (5-4)$$

where AB_0 is the initial response at the beginning of the dissociation curve, AB_{∞} is the final response once completely dissociated. Given k_{diss} value is 1.304 min^{-1} , K_D is thus determined to be 4.55 mM via $K_D = k_{\text{diss}}/k_{\text{assoc}}$.

5.3 Results and Discussion

5.3.1 Cavitand incorporated lipid membrane

Three cavitands were initially tested for their associative properties with the membrane bilayer; water-soluble tetracarboxylate cavitand **1**, and its more lipophilic counterparts **2** and **3**. The synthesis of **1-3** and their binding properties in both water and organic solvents have been previously reported^{10, 11, 35}. Cavitand **1** is soluble in water at

millimolar concentrations, whereas lipophilic cavitands **2** and **3** are only sparingly soluble in pure water, but can be incorporated into micelles in the presence of added lipid. All three hosts show millimolar affinity for choline and related trimethylammonium salts due to favorable cation- π interactions between the aromatic cavitand walls and the guest. In water, choline has a binding affinity for **1** of $2.6 \times 10^4 \text{ M}^{-1}$, although the K_A was lessened in an SDS micelle¹⁴. A significant advantage of cavitands as hosts is their open-ended character; long guests can extend out of the cavity, presenting large functional groups into the bulk solvent. This allows a variety of guest derivatives to be tested.

The experiment is illustrated in Figure 5.2. The membrane was deposited on a nanoglassified gold chip in a flow-cell¹⁹. The membrane was fabricated by the injection of preformed L- α phosphatidylcholine (PC) vesicles that fuse readily on this chip. Cavitands **1-3** were subsequently injected into the system in a 10% DMSO:H₂O solution (to maximize solubility) followed by a rinsing stage to remove all DMSO and unincorporated cavitand from the flowcell. Finally, a suitable guest (Figure 5.1c) was introduced to the system by injection, followed by copious rinsing to remove the unincorporated excess. PC was chosen as the constituent lipid to minimize the host:guest binding of the lipid inside the cavitand. The two alkyl chains in PC (oleoyl and palmitoyl) cannot both fit inside the cavity (as has been observed with SDS¹⁴), and the phosphate anion in the phosphocholine group minimizes binding at that terminus *via* a repulsive interaction with the carboxylates at the cavitand rim¹⁰.

The effect of addition of cavitand **1** to the membrane bilayer is shown by the SPR sensorgram in Figure 5.3. The change in resonance angle upon addition indicates the

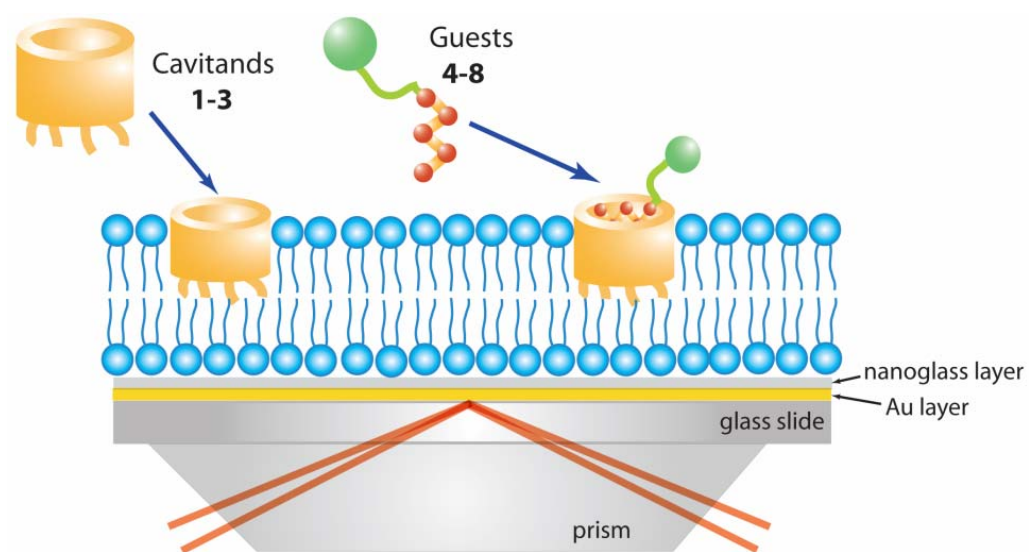


Figure 5.2. A cartoon representation of guests binding to cavitand **1** incorporated in a PC bilayer membrane.

binding of cavitand **1** to the PC membrane. The cavitand remains bound to the membrane bilayer even after extensive rinsing, and the angular shift indicates a surface coverage of $8.02 \pm 0.93 \times 10^{13}$ molecules/cm². The incorporation of cavitands **2** and **3** in the PC membrane was also observed, but with a substantially lower change in SPR signal and thus, surface coverage. We attribute this to their poor solubility in water and limited exposure to the membrane in the flowcell setting. All subsequent analyses were performed using water-soluble cavitand **1**.

The sensorgram does not, however, give any indication of the orientation of cavitand **1** in the membrane. To determine whether its binding properties remained intact, the cavitand:membrane system was exposed to the guest molecules shown in Figure 5.1c. To minimize nonspecific interaction between the guest and the membrane bilayer, commercially available long chain alkyltrimethylammonium salts were avoided. The guests used in this study were synthesized by the coupling of 2-(trimethylammonium)ethylamine chloride with either the isothiocyanate (**5,6**) or acid chloride (**7,8**) of the corresponding headgroup (Figure 5.1c). All showed good water solubility (≥ 20 mM), and were analyzed by ¹H NMR spectroscopy before injection.

5.3.2 Different guests bind to cavitand incorporated lipid membrane and binding affinity determination

Upon addition of choline **4**, no change in resonance angle was observed. This is not entirely unexpected, as molecular modeling (Figure 5.1b) indicates that choline is completely surrounded by the cavity upon binding, and does not protrude into the solvent above the membrane. The small change in the membrane composition upon small guest

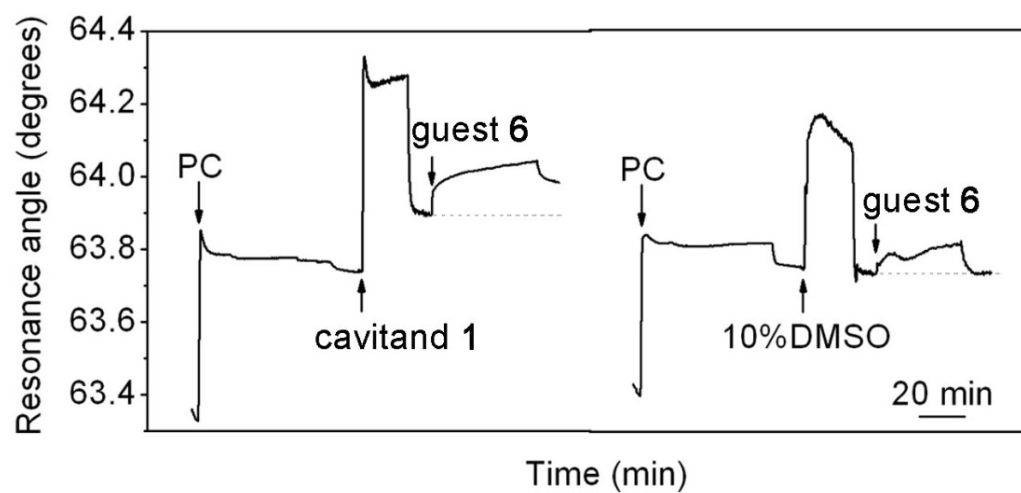


Figure 5.3. SPR sensorgrams for guest **6** interaction with PC bilayer membrane in the presence (left) and absence (right) of cavitand **1**.

binding would be difficult to detect, so host:guest affinity cannot be ruled out. Upon addition of larger guests **5-8** to the system, a small, reproducible change in resonance angle was observed, indicating guest binding and concomitant change in refractive index of the membrane. The SPR response to guest binding was dependent on the molecular weight of the substrate. SPR signal response increased with increasing guest molecular weight, and corresponding increase in perturbation of the environment at the membrane-water interface. The change in resonance angle for the three largest guests **6-8** was large enough to allow reasonably accurate calculation of K_D/K_A values, although this was not possible for smaller guests **4** and **5**.

The values of equilibrium dissociation constant (K_D) and binding affinity (K_A) for the host:guest interactions between guests **6-8** and cavitand **1** were determined from SPR sensorgrams via saturation binding mode and the results were listed in Table 5.1. Guest **6** showed millimolar binding affinity ($K_D = 3.54 \pm 0.92$ mM, $K_A = 282 \pm 73$ M⁻¹). This value is two orders of magnitude smaller than that observed for substituted trimethylammonium salts in pure water, but is consistent with the binding affinity observed under physiological conditions (phosphate buffer, as used in these experiments)¹⁰. For comparison, the transient SPR response method was also used to determine the binding constant to the cavitand:membrane complex. The K_D value (4.55 mM) agrees well with the number derived from equilibrium binding analysis. A stronger affinity was determined for biotin-derived guests **7** (K_D (**7•1**) = 1.87 ± 0.24 mM, $K_A = 535 \pm 68$ M⁻¹) and **8** (K_D (**8•1**) = 1.95 ± 0.61 mM, $K_A = 513 \pm 160$ M⁻¹). These binding affinities show that the cavitand retains most of its host abilities while incorporated in the

membrane. Furthermore, control experiments in the absence of cavitand **1** show no SPR response from addition of any of the guests **4-8** to the supported membrane. This indicates that there is no non-specific incorporation of the targets in the membrane even in the case of mildly lipophilic naphthalene guest **5**.

5.3.3 Fluorescent guest binding and fluorescence microscopy characterization

It should be noted that although the changes in resonance angle observed upon guest binding are small, the measurements are reproducible. Further evidence for guest incorporation was obtained by fluorescence microscopy. Construction of the membrane:cavitand **1**:guest **6** complex was performed as before, and the chip visualized under a confocal microscope. The fluorescence microscopic images are shown in Figure 5.4. After extensive rinsing, the boundary of the flow cell with **6** was clearly visible, demonstrating fluorescence signal from the guest **6**•cavitand **1** complex incorporated in the membrane bilayer. The host:guest system was strong enough to retain the substrate in the membrane for a period of many minutes under rinse conditions. In the absence of cavitand, little fluorescence was observed under the same excitation condition, providing further evidence that there was little nonspecific adsorption of **6** on the membrane. The result also illustrates the advantage of the choline-based guest system. Non-specific interaction between organic molecules and membrane bilayers is a significant problem when creating shape-based receptors for membranes. Hydrophobic substrates would be susceptible to incorporation in the membrane itself, but that is not observed in this case; negligible binding of the guests **4-8** can be observed in the absence of cavitand by either fluorescence or SPR.

Guest	M _w	K _A /M ⁻¹	K _d /mM
4	104.2	N/A	N/A
5	288.5	N/A	N/A
6	492.6	282 ± 73	3.54 ± 0.92
7	329.2	535 ± 68	1.87 ± 0.24
8	442.3	513 ± 160	1.95 ± 0.61

Table 5.1. Binding properties of guest molecules **4-8** in the PC•**1** complex.

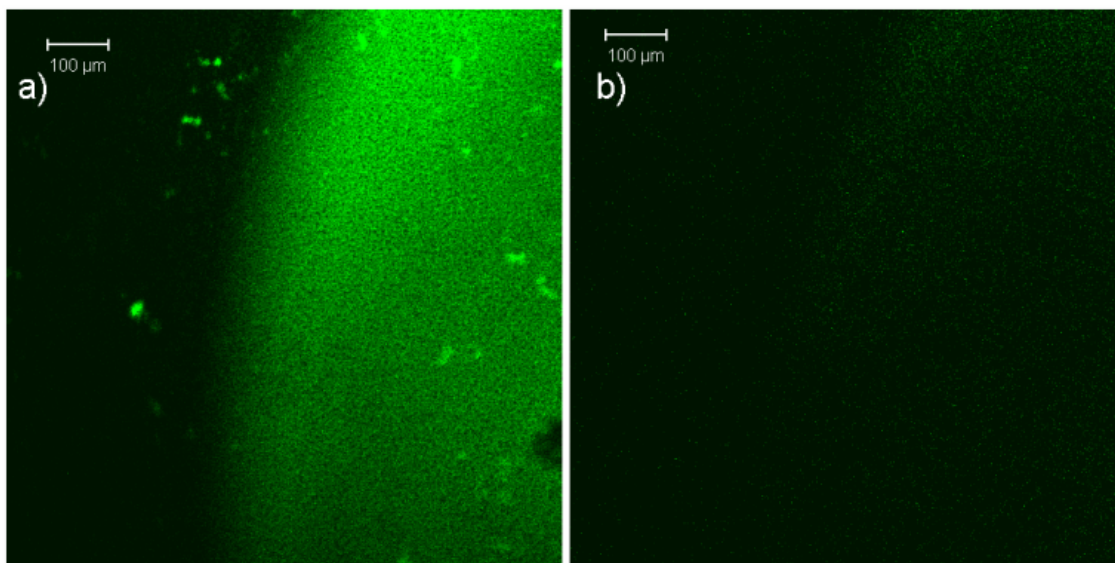


Figure 5.4. Fluorescence microscopic images of the calcinated chip containing a) PC bilayer, cavitant **1** and guest **6**; b) PC bilayer and guest **6** alone (control).

5.3.4 Biotin guest binding and neutravidin protein attachment

Cavitand **1** is able to non-covalently bind a variety of trimethylammonium-tagged species while incorporated in the supported membrane bilayer. The open-ended nature of the cavitand allows a wide scope of guest size; the trimethylammonium group is bound in the cavity, and the unencapsulated portion of the guest is displayed above the membrane surface.

Upon addition of NeutrAvidin to the preformed membrane:cavitand **1**:guest **7** complex, a significant change in resonance angle ($\Delta R = 0.20 \pm 0.02$ deg) was observed (Figure 5.5a), indicating that the complex is capable of displaying the biotin tag above the membrane while retaining its affinity for a suitable protein. As would be expected due to its much greater size and charge, NeutrAvidin displays a significantly larger ΔR upon binding than observed for the binding of guests **5-8**. Both biotin-derived guests **7** and **8** displayed affinity for NeutrAvidin. The sequential binding of cavitand, guest and NeutrAvidin are all observable. Addition of NeutrAvidin to the membrane:cavitand **1**:guest **8** complex caused a much larger change in resonance angle ($\Delta R = 0.42 \pm 0.06$ deg). Control experiments were performed by substituting biotin itself for guests **7/8**, and the addition of guests **7/8** to the membrane followed by NeutrAvidin in the absence of cavitand **1**. All controls were performed with the same concentration of guests. The sensorgram in Figure 5.5b displays no increase in resonance angle upon addition of biotin or NeutrAvidin, indicating minimal nonspecific interactions of the protein or biotin with the cavitand or the membrane itself. In the absence of cavitand **1**, (Figure 5.5c), neither guest **7** nor NeutrAvidin show significant incorporation in the membrane. Presence of

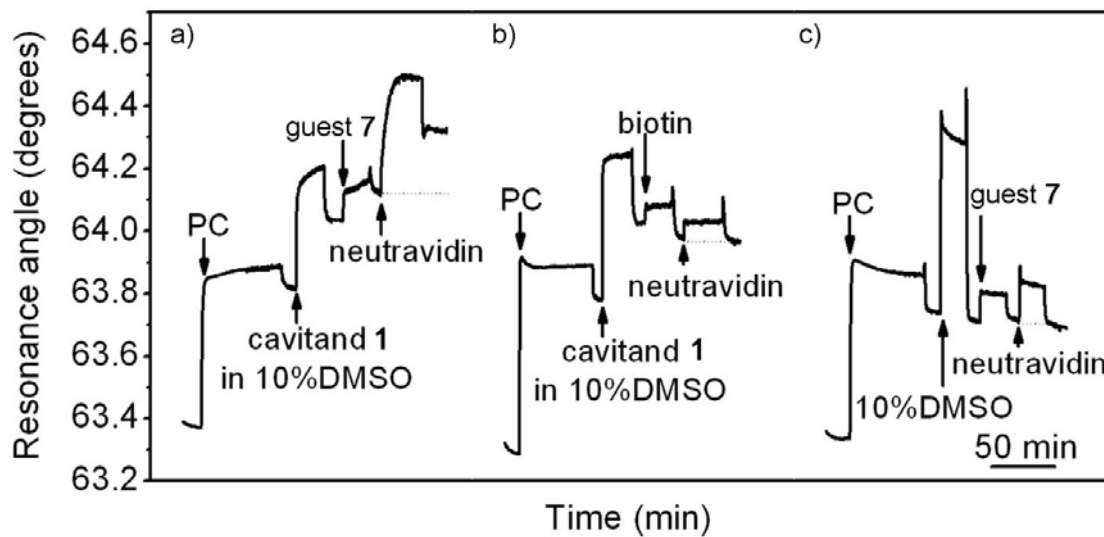


Figure 5.5. SPR sensorgrams for NeutrAvidin interaction with a supported PC membrane containing a) cavitand:guest complex **1•7**; b) cavitand **1** and biotin control; c) derivatized biotin guest **7** in the absence of cavitand.

both cavitand **1** and the trimethylammonium binding handle are required to attach the protein to the surface; a four-component binding event is seen, with only non-covalent interactions binding the cavitand, guest and NeutrAvidin to the supported membrane bilayer. The binding affinities are strong enough to hold the four-component complex together during the final washing phase.

The NeutrAvidin surface coverage was calculated by SPR response as before. When immobilized by the cavitand **1**•guest **7** complex, NeutrAvidin surface coverage was estimated to be $2.94 \pm 0.62 \times 10^{12}$ molecules/cm², (c.f. θ (cavitand **1**) = $8.02 \pm 0.93 \times 10^{13}$ molecules/cm²). The cavitand:NeutrAvidin ratio is 27.3: 1, which is to be expected due to the vast difference in sizes between cavitand **1** (MW = 1377 Da) and NeutrAvidin (MW= 60 kDa). The millimolar binding affinity of guest **7** will also contribute to this difference, as not all host molecules will be occupied. Although there was minimal change in the binding affinity for guests **7** and **8** in cavitand **1**, a significant difference in SPR response was detected upon NeutrAvidin binding. The surface coverage of NeutrAvidin when bound to the PC•**1**•guest **8** complex is $4.94 \pm 0.71 \times 10^{12}$ molecules/cm², which is two-fold greater than that observed for the shorter guest **7**. This is most likely due to reduced steric interactions between the protein and the membrane bilayer when the longer guest **8** is used as the recognition motif.

In addition to the surface coverage experiments, the binding affinity of NeutrAvidin for the cavitand•guest complex could be measured. This is unusual as the biotin:NeutrAvidin binding affinity is $\sim 10^{15}$ M⁻¹, far too high for analysis by SPR. In this case, the saturation binding mode gave the biotin:NeutrAvidin binding constant of $3.64 \times$

10^5 M^{-1} . This lowered binding affinity is most likely not due to the biotin:NeutrAvidin interaction, but the interaction between NeutrAvidin•7 complex and the membrane:cavitand complex – the “weakest link” is the cavitand:trimethylammonium interaction, and some of the guest may be pulled out of the cavitand upon NeutrAvidin coordination. Given that the binding affinity is at least partially driven by the hydrophobic effect³⁶, it is unsurprising that the affinity decreases upon conversion of the small 7 to the large, extremely hydrophilic NeutrAvidin•7 complex. The binding affinity was determined by regression analysis, and the best fit was obtained by assuming a 1:1 biotin:avidin binding motif. It is possible that multivalent binding between NeutrAvidin and the 1•8 complex can occur³⁷, but our binding data suggests this is not dominant in this case.

5.3.5 Molecular modeling for guest cavitand lipid membrane binding

Figure 5.6 shows an illustration of the relative sizes of the cavitand, guest and lipid bilayer (as determined by molecular modeling; SPARTAN, AM1 forcefield). The cavitand is relatively small, with a vertical height of approximately 13 Å. When compared to the POPC length (~26 Å), it becomes clear that the cavitand is most likely positioned towards the top of the lipid bilayer, incorporated in the first layer such that the negatively charged carboxylates are in proximity with the aqueous exterior, and the hydrophobic cavitand body is incorporated amongst the lipid hydrocarbon chains. This allows the host to present suitable guests to the exterior milieu.

The binding abilities of the membrane:cavitand:trimethylammonium complexes are quite remarkable – in the presence of lipids that can act as competitive substrates, in

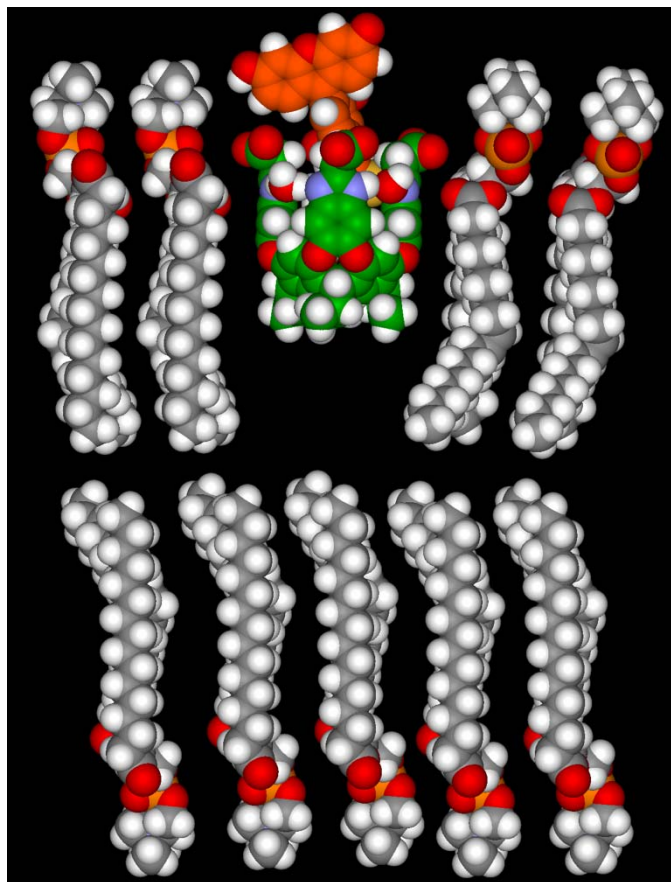


Figure 5.6. Illustration of the relative sizes of the cavitand **1**•guest **6** complex and POPC lipids (individual components minimized by SPARTAN, AM1 forcefield).

buffered solution that lowers substrate affinity and shear forces from the flowcell, a host the diameter of only *two* POPC lipid molecules is able to immobilize a 60 KDa protein. This protein is held in place solely by cation- π interactions between eight aromatic rings and a single trimethylammonium cation. Cation- π interactions are a vital component of protein:substrate recognition events³⁸, but this shows their potential as the “mortar” holding together quaternary nanoscale constructs.

5.4 Conclusions

In this work, we have shown that a deep water-soluble cavitand can be incorporated in a membrane bilayer attached to a nanoglassified surface, while still retaining its host properties. The open-ended nature of the cavitand allows binding of a variety of guest sizes; the trimethylammonium binding “handle” is incorporated inside the cavity while the rest of the molecule is displayed above the cavity in the aqueous phase. Competitive binding from the membrane lipids is minimal, and association constants on the order of 10^3 M^{-1} are observed with a variety of trimethylammonium-derived substrates. The binding events can be observed in real-time by SPR spectroscopy, and the binding of fluorescent derivatives observed by fluorescence microscopy. No non-specific binding of the guest molecules in the membrane itself can be observed in the absence of cavitand – the host can selectively recognize its targets in a biorelevant setting. The membrane:cavitand:guest complexes can subsequently be used to sense proteins at the membrane surface. By the use of a suitable biotin-derived guest molecule, the biotin motif is displayed above the membrane bilayer, and is able to immobilize NeutrAvidin at the surface, a process detectable by SPR. The surface coverage is dependent on the spacer

used to derivatize the biotin – increased distance from the bilayer allows a higher concentration of protein to be immobilized.

These binding studies showcase the potential of this system as a host for larger, more hydrophilic aggregates – the trimethylammonium:cavitand interaction is not only a product of the hydrophobic effect, so is tolerant to the binding of highly hydrophilic species such as proteins. Lipid/steroid “anchors” can be rendered useless if the external group is too hydrophilic (as has been observed for lipophilic oligonucleotides³⁹); exploiting non-hydrophobic interactions to anchor species in a membrane can allow a far greater range of target species. Further studies on the properties of membrane-bound hosts are underway.

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Chapter 6 Protein Recognition by a Self-assembled Deep Cavitand on a Gold Substrate

6.1 Introduction

The incorporation of synthetic host molecules on a substrate surface has been a goal of chemists for many years. By attaching well-known host molecules such as calixarenes, shallow cavitands and cyclodextrins to a gold or silica surface, detection of small molecules is possible¹. Many of these molecules are commercially available, and their ease of derivatization and surface attachment is an obvious attraction. They have been tested for self-assembly² and the detection of a range of small molecules including gas vapors³, polycyclic aromatic hydrocarbons *via* surface-enhanced Raman spectroscopy (SERS)⁴ and adrenaline and catecholamine *via* electro-chemistry⁵. More recently, improved vapor sensing has been shown with phosphonate cavitands that show much higher binding affinities for charged substrates than calixarenes or Cram-type cavitands⁶. These hosts, however, display rather small binding pockets. This leads to two challenges: recognition of larger and/or more flexible guests is challenging, and solvent competition can be problematic. One solution to this problem is multivalency: by adding substrates that can coordinate to *multiple* surface-bound species⁷, a variety of strongly bound substrates can be attached to surfaces, allowing for nanofabrication⁸ and the sensing and immobilization of biomolecules⁹.

While the field of surface attachment has focused on shallow, well-precedented hosts, significant advances have been made in the synthesis of solution-phase host species¹⁰. Deep cavitands are bowl-shaped molecules that feature sizable concave surfaces¹¹. By

appending flexible walls to the rim of the resorcinarene framework, larger cavities are possible at the cost of multiple conformation¹². Appending secondary amides¹³ or benzimidazoles¹⁴⁻¹⁶ along the periphery stabilizes the “vase-like” structure shown in Figure 6.1 by intramolecular hydrogen bonding¹⁷. These deep cavitands show wide-ranging properties, from molecular recognition¹⁸ to catalysis¹⁹ and selective stabilization of reactive intermediates^{20, 21}. Surface attachment of deep cavitands can provide a number of advantages over smaller, shallow hosts such as derivatized cyclodextrins and calixarenes^{3, 7-9}. Real-time analysis of guest binding from an aqueous medium is possible rather than detection of small organics through vapor absorption, and selective target immobilization is possible through *both* size/shape and charge-based non-covalent interactions.

Unfortunately, there are synthetic challenges in the formation of deep cavitands with suitable groups for surface attachment, so their use in surface chemistry has been limited. Here we describe the synthesis of a self-folding deep cavitand with thioether feet and its attachment to a gold surface. The surface coverage and guest binding properties can be monitored by surface plasmon resonance (SPR) spectroscopy and SPR imaging.

6.2 Experimental

6.2.1 Instrument

¹H spectra were recorded on a Varian Inova 400 or a Bruker DRX-600 spectrometer with a 5-mm QNP probe. Proton (¹H) chemical shifts are reported in parts per million (δ) with respect to tetramethylsilane (TMS, $\delta=0$), and referenced internally with respect to the protio solvent impurity. Deuterated NMR solvents were obtained from Cambridge

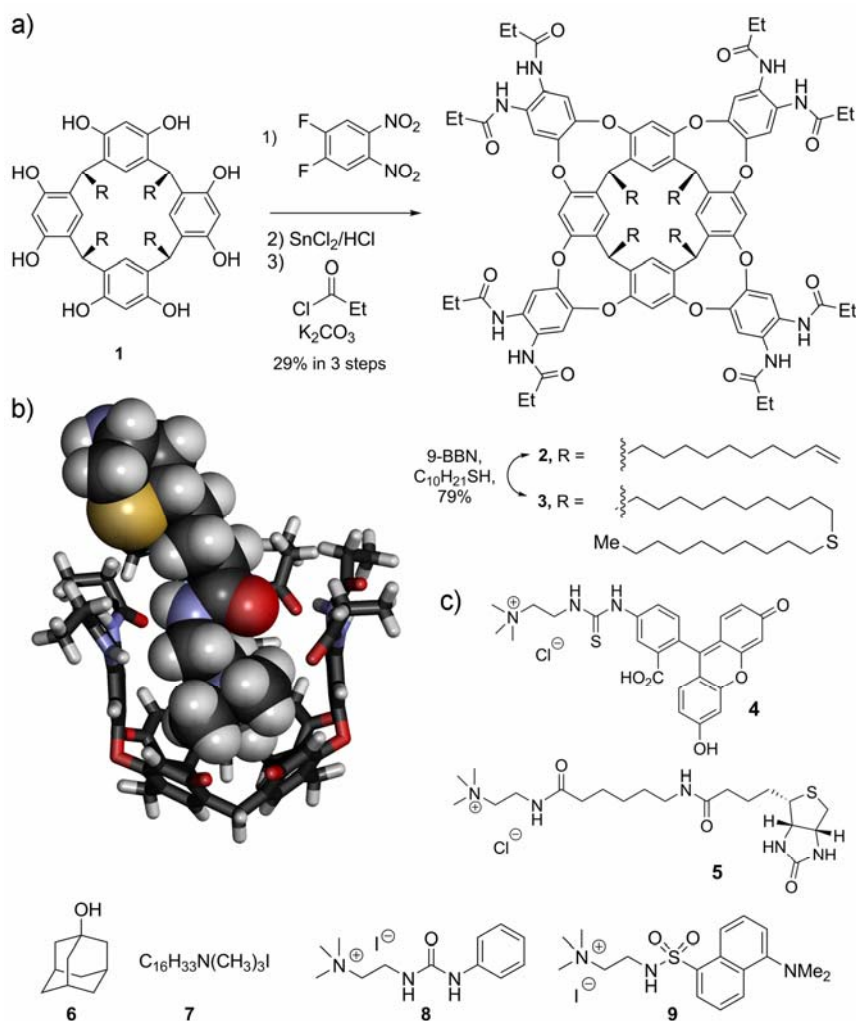


Figure 6.1. a) Synthesis of cavitands **1-3**; b) Minimized conformation of **1** (SPARTAN, AM1 forcefield) with a NMe_3^+ -tagged guest in the cavity; and c) the guests used in this study.

Isotope Laboratories, Inc., Andover, MA, and used without further purification. All other materials were obtained from Aldrich Chemical Company, St. Louis, MO and were used as received. Solvents were dried through a commercial solvent purification system (SG Water, Inc.). Cyclic voltammetric experiments were performed on a CHI 650A Electrochemical Workstation (CH Instruments Inc.). All experiments were carried out using a conventional three-electrode system with the gold disc electrode as working, a platinum foil as auxiliary, and a saturated calomel electrode as reference. A dual channel SPR spectrometer NanoSPR-321 (NanoSPR, Addison, IL) with a GaAs semiconductor laser light source ($\lambda=670\text{nm}$) was used for all SPR measurements. The device comes with a high-refractive index prism ($n = 1.61$) and 30 μL flow cell. Surface interaction and modification were monitored using the angular scanning mode around the minimum angle. The modified gold substrate was clamped to a flow cell on a prism. Fluorescence microscopy was carried out on a Zeiss LSM 510 confocal laser scanning microscope with 488 nm argon laser excitation and a CCD camera. The synthesis of resorcinarene **1**²² and guests **4** and **5**²³ was performed according to literature precedent.

6.2.2 Cavitand synthesis

Octaamide cavitand 2

1) To a solution of resorcinarene **1** (6.0 g, 4.3 mmol) and difluorodinitrobenzene (4.8 g, 23.6 mmol) in DMF (180 mL) was added Et_3N (12.6 mL, 90.6 mmol) at 0 °C. The solution was heated at 70 °C and stirred for 24 h. The resulting mixture was poured into 1.5 L of water containing 2M HCl (30 mL) and the precipitate was collected by filtration

and washed with water. The product was extracted with EtOAc and dried with MgSO₄. Evaporation of the solvent gave crude octanitrocavitand as a brown solid (13.6 g). This compound was used for next reaction without further purification.

2) To a solution of octanitro cavitand (3.0 g) in ethanol (80 mL) and concentrated HCl (20 mL) was added tin(II) chloride dihydrate (23g, 102 mmol). The mixture was stirred at 70 °C for 22 h and then allowed to evaporate *in vacuo* to give the crude octaamine cavitand. This compound was used for next reaction without further purification.

3) To a solution of octamine cavitand in EtOAc (400 mL) was slowly added aqueous solution (300 mL) of K₂CO₃ (51 g, 369 mmol) and propionyl chloride (20 mL, 229 mmol) at room temperature. The reaction mixture was stirred at room temperature for 2 h. The precipitate was filtrated and washed with EtOAc. The filtrate was dried with Na₂SO₄ and evaporated to give a crude product. Flash chromatography (SiO₂, hexane/EtOAc = 2:2, *R*_f = 0.34) afforded octaamide cavitand **2** (697 mg, 0.37 mmol, 29% in 3 steps) as a white solid. ¹H NMR (600 MHz, acetone-*d*₆) δ 9.48 (s, 8H, NH), 7.89 (s, 4H, ArH), 7.70 (s, 8H, ArH), 7.48 (s, 4H, ArH), 5.78-5.86 (8H, ArCH and CH=CH₂), 5.00 (dd, 4H, *J* = 17.2, 1.6 Hz, *cis*-CH=CH₂), 4.92 (dd, 4H, *J* = 10.2, 1.0, *trans*-CH=CH₂), 2.52 (m, 8H, ArCHCH₂), 2.42 (m, 16H, COCH₂), 2.06 (m, 8H, CH₂CH=CH₂), 1.28-1.50 (48H, CH₂), 1.22 (t, 24H, *J* = 7.6 Hz, CH₃); ¹³C{¹H}NMR (150 MHz, acetone-*d*₆) δ 174.3(C=O), 155.7 (aromatic), 150.3 (aromatic), 139.8 (CH=CH₂), 136.9 (aromatic), 129.4 (aromatic), 125.8 (aromatic), 121.7 (aromatic), 117.0 (aromatic), 114.7 (CH=CH₂), 34.5 (CH₂), 34.4 (CH₂), 32.9 (CH₂), 31.3 (CH₂), 30.5 (CH₂), 30.4 (CH₂), 30.3 (CH₂), 29.9 (CH₂), 29.8 (CH₂), 10.6 (CH₃); HRMS (ESI-TOFMS: MH⁺) calcd. for C₁₁₆H₁₄₅N₈O₁₆⁺ 1906.0773, found 1906.0731.

Sulfide-footed deep cavitand **3**

9-Borabicyclo[3.3.1]nonane (9-BBN) (1.2 mL, 0.6 mmol, 0.5 M in THF solution) and 1-decanethiol (1.1 mL, 5.3 mmol) were added to a solution of **9** (550 mg, 0.29 mmol) in THF (100 mL) at 0 °C. The solution was stirred at ambient temperature for 23 h. After evaporation of the solvent under reduced pressure, the crude product was extracted with hexane to give a sulfide-footed octaamido cavitand **3** as white solid (604 g, 0.23 mmol, 79%). ¹H NMR (600 MHz, acetone-*d*₆) δ 9.48 (s, 8H, NH), 7.90 (s, 4H, ArH), 7.71 (s, 8H, ArH), 7.49 (s, 4H, ArH), 5.84 (4H, ArCH), 2.52 (24H, SCH₂, ArCHCH₂), 2.42 (m, 16H, COCH₂), 1.58 (m, 16H, SCH₂CH₂), 1.28-1.50 (48H, CH₂), 1.22 (t, 24H, *J* = 7.6 Hz, CH₃), 0.89 (t, 16H, *J* = 7.0 Hz, CH₃); ¹³C{¹H}NMR (150 MHz, acetone-*d*₆) δ 174.3 (C=O), 155.7 (aromatic), 150.3 (aromatic), 136.9 (aromatic), 129.4 (aromatic), 125.8 (aromatic), 121.7 (aromatic), 117.1 (aromatic), 34.5 (CH₂), 33.0 (CH₂), 32.7 (2CH₂), 31.3 (CH₂), 30.6 (2CH₂), 30.5 (CH₂), 30.4 (CH₂), 30.1 (2CH₂), 29.7 (CH₂), 29.6 (CH₂), 29.0 (CH₂), 27.9 (CH₂), 27.7 (CH₂), 27.5 (CH₂), 27.2 (CH₂), 23.4 (CH₂), 22.9 (CH₂), 14.5 (CH₂), 10.6 (CH₃); MS (MALDI-TOFMS: MNa⁺) calcd. for C₁₅₆H₂₃₂N₈O₁₆S₄⁺ 2624, found 2625.

6.2.3 Surface preparation and characterization

Preparation of Cavitand **3** Self-Assembled Monolayer (SAM)

A gold disc electrode (CH Instruments Inc., 2.0 mm in diameter) was abraded with fine silicon carbide paper and polished carefully with 0.3 μm and 0.05 μm alumina slurry, and then sonicated in water. The cavitand **3** self-assembled monolayer was prepared by immersing the cleaned gold electrode in a 0.5 mM butanol solution of cavitand **3** for 5 hours, followed by washing with ethanol and distilled water.

Establishing Complete Cavitand 3 SAM with space-filling molecules

N-octadecanethiol (ODT) and 11-mercaptoundecanoic acid (MUA) were chosen to fill the defects in the SAM left by cavitand absorption and mixed with cavitand **3** in butanol solution with molar ratio 10:1 (5 mM ODT or MUA, 0.5 mM **3**) respectively. SPR gold chips were fabricated with a 2nm thick chromium adhesion layer, followed by deposition of a 46 nm thick gold layer via e-beam evaporation onto cleaned BK-7 glass slides. To prepare the mixed SAM of **3** and thiols ODT/MUA, gold substrates were immersed in the above butanol solution for 5 hours, followed by washing with ethanol and distilled water.

Electrochemical measurements

Cyclic voltammetric experiments were performed on a CHI 650A Electrochemical Workstation (CH Instruments Inc.). All experiments were carried out using a conventional three-electrode system with the gold disc electrode as working, a platinum foil as auxiliary, and a saturated calomel electrode as reference electrodes. The redox solution consisted of 25mM aqueous KCl containing 2.5mM $[\text{Fe}(\text{CN})_6]^{3-}$ with a scan rate of 100 mV/s.

SPR analysis of guest binding on SAMs

A dual channel SPR spectrometer NanoSPR-321 (NanoSPR, Addison, IL) with a GaAs semiconductor laser light source ($\lambda=670\text{nm}$) was used for all SPR measurements. The device comes with a high-refractive index prism ($n = 1.61$) and 30 μL flow cell. Surface interaction and modification were monitored using the angular scanning mode around the

minimum angle. The modified gold substrate was clamped to a flow cell on a prism. An aqueous solution of **4** (5 mM) was injected into the flow cell and incubated 30 min to allow guest binding to **3**.

Protein binding of the cavitand:guest construct was measured similarly. A 2 mM aqueous solution of biotin guest **5** was injected into the flow cell and incubated on the 10:1 MUA:Cavitand **3** SAM gold substrate for 30 min followed by nanopure water rinsing. An aqueous solution of streptavidin (0.25 mg/mL) was injected into the flowcell, and the change in resonance angle recorded. The experiment was repeated on a blank MUA SAM, and the change in resonance angle upon streptavidin injection compared to that in the presence of **3**.

Fluorescence microscopy characterization

After the 10:1 MUA:**3** SAM gold substrate was incubated with 5mM **4** and rinsed with nanopure water, fluorescence microscopy was carried out on a Zeiss LSM 510 confocal laser scanning microscope with 488 nm argon laser excitation and a CCD camera. Weak signal was observed due to fluorescence quenching from the gold surface.

Array construction and SPR imaging characterization

PDMS was prepared by mixing 10:1 prepolymer and curing agent and degassed for 20 min. The mixed solution was poured to a petri dish and incubated at 70 °C in an oven for 1 hour. The resultant PDMS chip was carefully peeled off and drilled with a homemade tool to generate a 2 x 2 array. PDMS array was tightly pressed on a plasmon cleaned gold substrate. After incubation in an oven at 70 °C for 20 min, a butanol solution of 10:1 MUA:**3** was pipetted on array cavities and incubated for 3 hours. The solution was added

several times during incubation to replenish solvent loss during evaporation. After the deposition, the array was patted dry with a Kimwipe and rinsed with ethanol. The PDMS array chip was peeled off and the gold substrate was immersed in a 5mM solution of **4**. After rinsing with nanopure water, the array was subjected to SPR imaging characterization.

The prepared array was mounted on an optical stage with a flow cell for SPR imaging. A red light emitting diode (LED, 648 nm) was used for excitation and the reflected images were captured by a cooled 12-bit CCD camera (Retiga 1300 from QImaging) with a resolution of 1.3 MP (1280 x 1024 pixels) and 6.7 μm x 6.7 μm pixel size. The guest binding on the substrate surface was monitored by measuring changes in the reflectivity, and real time images were recorded.

6.3 Results and Discussion

6.3.1 Self-assembly monolayer of cavitand on gold substrate

The synthesis of the target cavitand **3** is shown in Figure 6.1a. Facile adsorption on a gold surface requires the presence of thiol or sulfide groups on the receptor, and obviously these should be incorporated at the base (or “feet”) of the receptor. Introduction of thiols or thioacetates from known cavitands with alcohol feet proved problematic, so we turned to a precedented method of resorcinarene derivatization, by reacting a suitable thiol with alkene groups at the resorcinarene base²². Olefinic resorcinarene **1** was converted to the octamide cavitand **2** in 29% overall yield (3 steps) by reaction with excess 1,2-difluoro-4,5-dinitrobenzene, followed by SnCl_2/HCl reduction and acylation under Schotten-Baumann conditions¹⁷. It should be noted that the

terminal olefins are stable to these reduction conditions, although other reported reductions (e.g. Raney Nickel/H₂) are unsuitable. The addition of thiol groups was performed after this stage as the RSR function does not survive the harsh reduction conditions necessary for synthesis of octamide **2**. Hydroboration of the olefins in **2** with 9-BBN in the presence of 1-decanethiol proceeded smoothly, furnishing the target sulfide-footed cavitand **3** in 79% yield after column chromatography. The cavitand is soluble in a wide variety of organic solvents (although not water), and *n*-butanol was chosen as a suitable solvent for surface deposition to limit damage to the cleaned surface while allowing sufficiently slow evaporation for clean surface formation. In order to allow real-time surface plasmon resonance (SPR) spectroscopy analysis of the system, gold-plated electrodes were chosen as the target surface. SPR is a surface analysis method based on detecting refractive index changes due to the adsorption or non-covalent recognition of target molecules, and is useful for monitoring affinity interactions in real-time *without* the use of a label. SPR gold chips were fabricated with a 2nm chromium adhesion layer, followed by deposition of a 46 nm gold layer via e-beam evaporation on pre-cleaned BK-7 glass slides.

Different self-assembly methods

Figure 6.2 demonstrated three different methods to generate self assembly monolayer. As for cavitand alone self assembly monolayer, the receptor was deposited onto the cleaned chip by immersing the cleaned gold electrode in a 0.5 mM solution of cavitand **3** in *n*-butanol for 5 hours, followed by rinsing and drying. Electrochemical analysis was

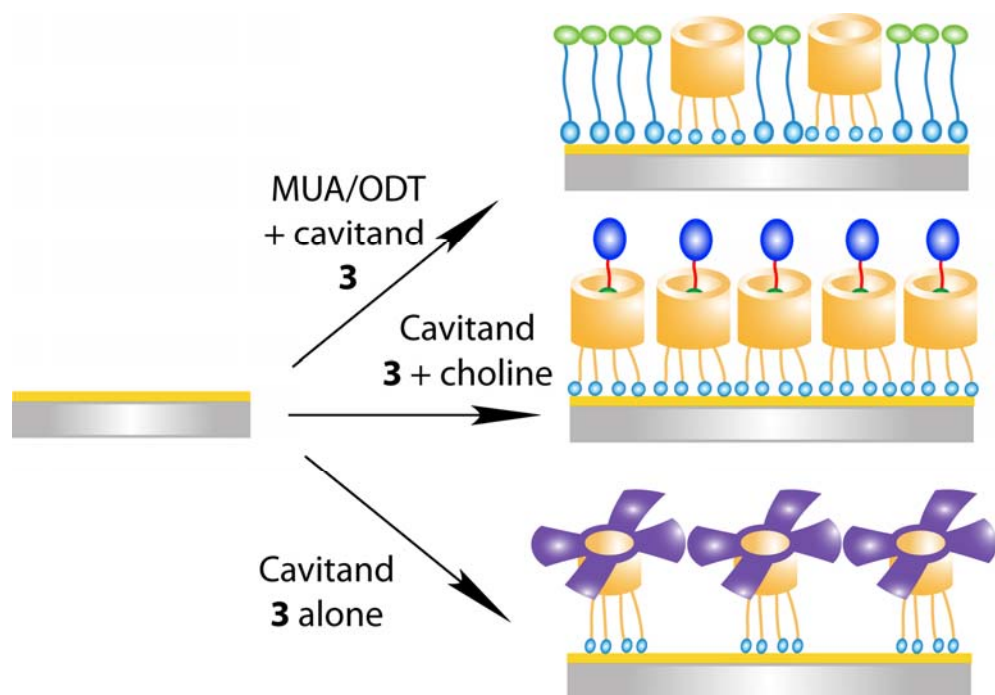


Figure 6.2. Illustration of cavitand **3** adsorption on the surface in the presence of a) space-filling molecules MUA or ODT, b) choline chloride and c) no additive.

used to determine the successful adsorption of **3** on the surface. Figure 6.3 shows the cyclic voltammograms of the bare and modified electrodes in 25mM KCl solution containing 2.5mM $[\text{Fe}(\text{CN})_6]^{3-}$ as charge carrier. Well-defined redox peaks were observed for the facile probe of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ on a bare surface. The presence of cavitand **3** caused a 63% decrease in current, indicating an impeded electron transfer on the electrode surface. The increase in peak separation, ΔE_p , from 90mV to 194mV, further points to a blocked process when the thiocavitand was assembled on the surface. The retention of some signal in the cyclic voltammogram, however, indicates that simple adsorption of the sulfide cavitand **3** did not cause complete coverage of the plate. The flexible cavitand **3** did not form a fully packed self-assembled monolayer, most likely due to poorly favorable interactions between cavitands at the surface. There are two possible conformation of the octamide cavitand, the folded “vase” conformation seen in Figure 6.1, and a flattened “kite” conformation with no cavity. This kite conformation is disfavored in aprotic solvents, as the cavitand is held in the desired “vase” conformation by the seam of hydrogen bonding amides at the rim. This seam is disrupted in protic solvents such as butanol, and the host undergoes rapid equilibration between the folded vase form and the unfolded kite conformation^{24, 25}.

Self-assembly of cavitand 3 with choline chloride incorporation

To corroborate the theory that unfolded conformations of **3** are responsible for the incomplete coverage, the deposition experiment was performed in the presence of a good guest molecule. Strongly bound species such as tetramethylammonium cations or choline have been shown to template the self-assembly of amide-based cavitands in protic or

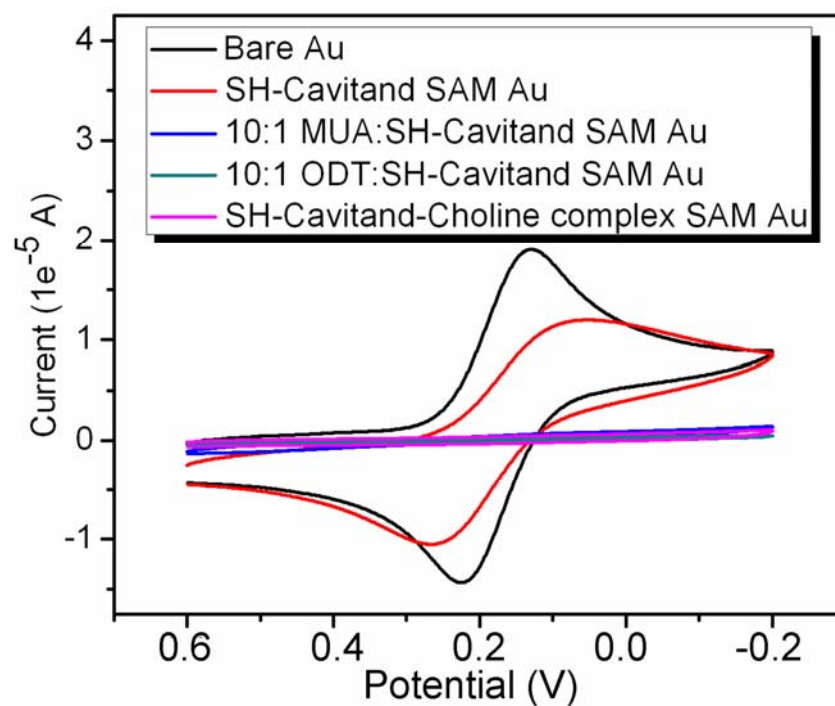


Figure 6.3. Cyclic voltammetry characterization of various surface coverages; charge carrier 2.5 mM $\text{K}_3\text{Fe}(\text{CN})_6$ in 25 mM KCl at 100mV/s.

aqueous solution and favor the “vase” conformation²⁵. Choline chloride (5 mM) was added to the 0.5 mM solution of **3**, and deposited on the cleaned gold electrode as before. After rinsing and drying, the cyclic voltammogram was recorded (Figure 6.3). In the presence of strong guest, no signal was observed in the CV, indicating formation of a SAM that completely shuts down the electronic communication due to a packed surface structure.

Self-assembly of cavitand with space filling molecules MUA or ODT

Unfortunately, while adsorption of the cavitand was successful in the presence of a strong guest, the presence of choline in the system removes the possibility of directed guest binding by **3** at the gold surface, as the cavity of **3** is filled. An alternate possibility is to “fill the gaps” with a suitable space-filling molecule that has no cavity. Even though a close-packed monolayer of empty cavitand **3** was inaccessible, the spaces between receptors need not be filled with other receptor molecules, but rather with simple molecules whose sole task is to fill the remaining defects in the monolayer. To prevent unwanted interactions between added guests and defects in the monolayer, films were also made by adsorbing a mixture of **3** and long chain aliphatic thiols.

Two thiols were tested, hydrophobic N-octadecanethiol (ODT) and hydrophilic mercaptoundecanoic acid (MUA), where the narrow alkanethiols can fill in gaps in the monolayer. Formation of the mixed monolayers was performed using the same method of cavitand adsorption. The gold chips were immersed in a 10:1 mixture of **3** and the requisite thiol as before, and surface coverage was determined by cyclic voltammetry (Figure 6.3). Both thiols were successful in forming a fully packed monolayer. In each

case, no signal was observed in the voltammogram, indicating formation of a SAM that completely shuts down the electronic communication due to a packed surface structure. The CV data does not indicate whether mixed monolayers were formed, however; this was determined by analysis of guest binding.

6.3.2 Guest Recognition

Having incorporated the host on the gold surface, the unanswered question was whether the cavitands retained their guest binding properties. Guest binding could be limited by unfolded conformations of cavitand in the **3** monolayer (in the absence of choline template), and MUA or ODT could outcompete **3** in surface adsorption, leading to zero cavitand at the **3**:ODT or **3**:MUA surface. The guest recognition properties of the three surfaces were established by monitoring the recognition event with guest molecules in real time by SPR analysis. The hosts are open-ended, and are well-precedented to bind substituted trimethylammonium (TMA) salts in both aprotic and aqueous solvents^{16, 26, 27}. The trimethylammonium tag acts as an anchor, and a variety of species can be displayed above the rim of the receptor.

The cavitand-adorned gold substrate was clamped down by a flow cell on a high-refractive index prism for SPR measurement, allowing exposure of the surface to aqueous medium. The flowcell apparatus allowed the simple introduction of suitable guest molecules by injection of targets in aqueous solution. Cavitand **3** displays a hydrophobic cavity to the aqueous milieu above the surface, capable of binding suitably

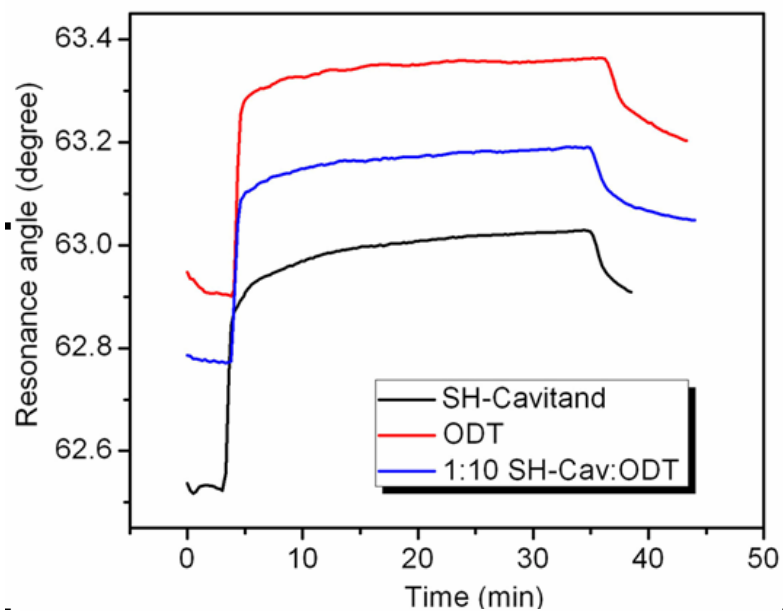
sized guests through hydrophobic and cation- π interactions. To this end, we utilized a series of guests that had been previously shown to bind in a membrane-bound deep cavitand in aqueous solution²⁵, as shown in Figure 6.1.

Fluorescein **4 guest binding on cavitand **3** surface with filling molecule MUA and ODT**

TMA-tagged fluorescein **4** was selected as the initial test subject. An aqueous solution (5mM) of guest **4** was injected into the chip-containing flow cell and incubated for 30 min to allow complete guest binding to **3**, followed by an aqueous rinsing process to remove the unincorporated excess. Initially, the cavitand **3** SAM and the **3**:ODT surfaces were exposed to guest, along with a control surface of ODT alone. The change in resonance angle upon treatment of the three derivatized surfaces is shown in Figure 6.4. An increase in resonance angle of 0.40° is observed upon addition of guest to the cavitand **3** SAM, indicating absorption of guest **4** to the surface. The signal increase persists after washing, and **4** has no affinity for the bare gold surface itself. The absorbed guests are held in place by hydrophobic and cation- π interaction with the cavity of **3**, and the fluorescein tag is displayed above the cavitand at the surface.

While the surface-adsorbed **3** retains its solution-phase binding properties, the efficacy of the space-filling strategy was undetermined. Guest **4** was initially injected to the chip containing the hydrophobic **3**:ODT mixed SAM. When **4** was exposed to the **3**:ODT mixed SAM, a smaller change in resonance angle (0.27°) occurred than for that of **3** alone. This indicates adsorption of the guest to the SAM, and the smaller signal would be expected for a surface with a lower concentration of host **3**. Unfortunately, significant

	SH-Cav	ODT	1:10 SH-Cav:ODT
Fluorescent guest	0.40 (± 0.06)	0.26 (± 0.02)	0.27 (± 0.01)



	SH-Cav	MUA	1:10 SH-Cav:MUA
Fluorescent guest	0.40 (± 0.06)	0.04 (± 0.01)	0.16 (± 0.03)

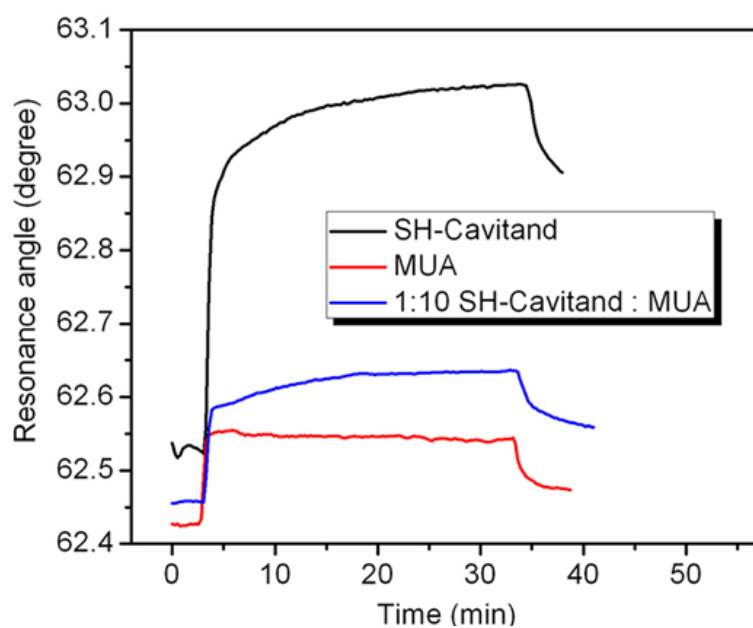


Figure 6.4. Sensorgrams showing the SPR response upon addition of guest **4** to the representative surfaces.

background absorption was observed in the ODT monolayer. The SPR signal increase upon addition of **4** is similar (0.26°) for a single ODT monolayer as the signal increase for the 10:1 ODT-**3** mix. Two possibilities present themselves: either ODT outcompeted **3** for surface attachment, or the affinity of **4** for the ODT monolayer itself is too high. While guest **4** is charged and water soluble, it is still quite lipophilic, and significant absorption occurs at the hydrophobic SAM chains.

To suppress the background absorption, the more hydrophilic 11-mercaptoundecanoic acid (MUA) was used as filler. The carboxylic acid groups are oriented toward the aqueous medium, lowering the lipophilicity of the SAM and limiting the background absorption due to non-specific hydrophobic adsorption. A self-assembled monolayer of MUA was constructed, and the affinity of guest **4** for MUA itself was determined. Upon treatment of the MUA SAM with an aqueous solution of guest **4**, a negligible SPR signal increase (0.04°) was observed, indicating minimal affinity of guest **4** for the surface. When guest **4** was exposed to the 10:1 **3**:MUA SAM, however, a strong signal increase was observed (0.16°). Attempts were made to corroborate the SPR experiment using fluorescence microscopy. The fluorescent guest **4** was obviously used for this analysis, but unfortunately the fluorophore exhibited significant quenching from the gold surface, and only very weak signal was observed in the experiment.

SPR characterization of guests binding on cavitand **3 : MUA surface**

Deep cavitands such as **3** show a wide scope of guest binding in solution, but this is lessened significantly in competitive solvents (i.e. those that can fill the cavity) and aqueous solution. At the surface, another challenge is raised - the guest must be of

Guest	4	5	6	7	8	9
Θ (3:MUA,9:1)	0.16	0.04	0	0.02	0.05	0.06
Θ (MUA)	0.04		0	0	0.02	0.02

Table 6.1. SPR Resonance angle change upon guest binding

suitable size and hydrophobicity to cause a sufficiently large refractive index change for good resolution in the SPR experiment. To analyze the range of species that could be detected in the system, we generally focused on water-soluble guests of varying sizes with trimethylammonium anchors. The guests are shown in Figure 6.1 and were all injected into the system as 5 mM aqueous solutions. As would be expected, all guests **5-9** showed smaller signal SPR increases upon binding in the **3**:MUA monolayer than fluorescein guest **4** due to their smaller size (Table 6.1). The binding of adamantanol **6** was not observed by SPR, but this is likely due to poor detection sensitivity rather than poor binding, as adamantyl groups are well-precedented guests for octamide cavitands in solution¹⁰. Interestingly, the observed binding for cetyltrimethylammonium bromide was poor; the highly hydrophobic alkyl chains should cause significant resonance angle change upon binding, but little was observed. This is most likely due to the unfavorability of guest binding - the hydrophobic penalty upon display of the C₁₆ chains to the aqueous medium outweighs any enthalpic benefit from NMe₃⁺ binding in the cavity. Changes in resonance angle were observed upon binding of other, less hydrophobic NMe₃⁺-tagged guests **5** and **7-9**. All four guests were immobilized at the surface and the signal increase was concomitant with the size of the headgroup. The changes in resonance angle are small, but reproducible, and only minimal background signal change was observed, indicating the selectivity of the system for suitably tagged targets.

6.3.3 SPR Imaging Analysis of Cavitand Arrays

The simple adhesion of cavitand **3** at the surface suggested the possibility of forming defined arrays of cavitand on the gold surface. Microarrays have been proposed as

powerful tools for routine screening and high-throughput tests in medical and biological field. On account of their scalable and cost-effective fabrication, microarray resonators are particularly well-suited for multiplexed diagnostic applications. We applied the self-assembly of the cavitand 3: guest interaction based on the microarray format, which provides *spatial* information on a sensing chip for binding sites as well quantitative assessment of the interaction. The application of microarray based technique in studying cavitand-guest interaction holds great promises for parallel evaluation of affinity for various guests and opens the door to large-scale screening of targets in the fields of drug discovery and pharmaceuticals.

Implementation of these analytical tools often relies on signal amplification strategies to reach the satisfied sensitivity levels. Fluorescence-based readout systems are by far the most widespread. However, common fluorescence-based microarray requires preliminary attachment of a quantifiable fluorescent tag and has limited application for on-line detection, not to mention the quenching we observed in our preliminary experiments (*vide infra*). We therefore turned to SPR imaging (SPRi) for array analysis. SPRi retains the high sensitivity of label free SPR measurement while providing spatial information of a sensing surface suitable for microarray detection. SPRi measures the intensity change of the reflective light at a fixed angle, and the image of the entire biochip can be recorded with high spatial resolution in real time²⁸. Coupled with microarray-based techniques, multiple microRNA sequences have been detected at low concentrations²⁹, and rapid detection of relevant protein biomarkers and nucleic acids with is possible by combining SPRi and enzymatic reaction amplification³⁰. Beside nucleic acids, lectin-carbohydrate

interactions³¹ and lipid-protein interaction was also studied via SPRi on bilayer lipid membrane arrays from photolithography³² and poly(dimethylsiloxane) (PDMS) microchips³³.

In order to prepare the array of host molecules on the surface, a PDMS stamp was prepared. PDMS polymer was freshly synthesized and a 2x2 array was drilled into the stamp. The stamp was pressed onto the freshly cleaned gold surface. After heated incubation, a butanol solution of 10:1 MUA:**3** was pipetted onto the array cavities. Butanol was chosen as solvent to limit overly rapid evaporation during deposition of the cavitand:MUA array as before; ethanol was unsuitable for this reason, and hydrocarbon solvents suffered from leakage from the PDMS stamp. After the adsorption had occurred, the system was dried and rinsed with ethanol, followed by removal of the PDMS stamp from the chip.

SPR imaging of these arrays requires a significant change in resonance angle for suitable sensitivity. Upon formation of the **3**:MUA monolayer, only minimal changes in signal were observed between array and background. As SPR spectroscopy is effective for detecting guest binding, we attempted to attain SPR image data on the host:guest complex rather than the **3**:MUA monolayer alone. The preformed array was incubated with a 5 mM aqueous solution of fluorescent guest **4** for 30 mins at ambient temperature, and the chip exposed to SPR imaging characterization. In this case, the resolution of the cavitand:**4** host guest complex was sufficient for good SPR imaging analysis.

Imaging of the array is shown in Figure 6.5. An increase in reflected light intensity was observed upon binding of guest **4** that only occurred in the 2x2 array. Figure 6.5 shows

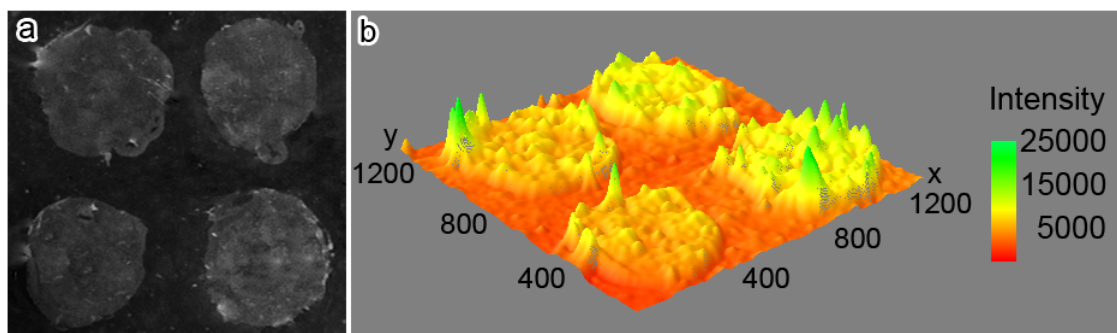


Figure 6.5. a) SPR imaging of fluorescent guest **4** incubated on a 2x2 array of cavitand **3** (4mm in diameter); b) corresponding intensity 3D profile.

two views of the array, a plan view of the light/dark profile (Figure 6.5a), and a 3D profile indicating signal intensity (Figure 6.5b) that gives better spatial resolution. The gold chip was plasma cleaned before use and remained hydrophilic after removal of the PDMS stamp, and therefore provided little nonspecific adsorption for fluorescent guest **4** outside the array boundary. This data shows that not only is the sulfide-footed cavitand **3** amenable to surface deposition, it is possible for defined arrays of the system. The guest binding properties can be determined in a label-free manner in mild conditions - although fluorescein-derived guest **4** was used for this purpose, the detection is not based upon fluorescence. Any NMe_3^+ -tagged guest of suitable size is amenable to this process.

6.3.4 Protein Recognition

The cavitand monolayer has excellent substrate selectivity: suitable targets are substituted NMe_3^+ ions. In order to widen the scope of the system, tagged guests that *themselves* contain a recognition element are desired. Extended cap-biotin guest **5** can be attached to the surface by interaction of the trimethylammonium tag with cavitand **3**, and the biotin group displayed above the surface for immobilization of biomolecules. Sequential injection of guest and streptavidin protein allows monitoring of the biosensing power of the system.

The **3**:MUA surface was utilized for this experiment. After immersion of the gold chip in a 10:1 solution of MUA:**3** followed by rinsing with butanol and water, the modified gold substrate was dried under a N_2 stream and clamped to a flow cell on an SPR prism. A 2 mM aqueous solution of cap-biotin guest **5** was injected into the flow cell and monitored by SPR analysis. Upon binding of **5**, a change in resonance angle of 0.039°

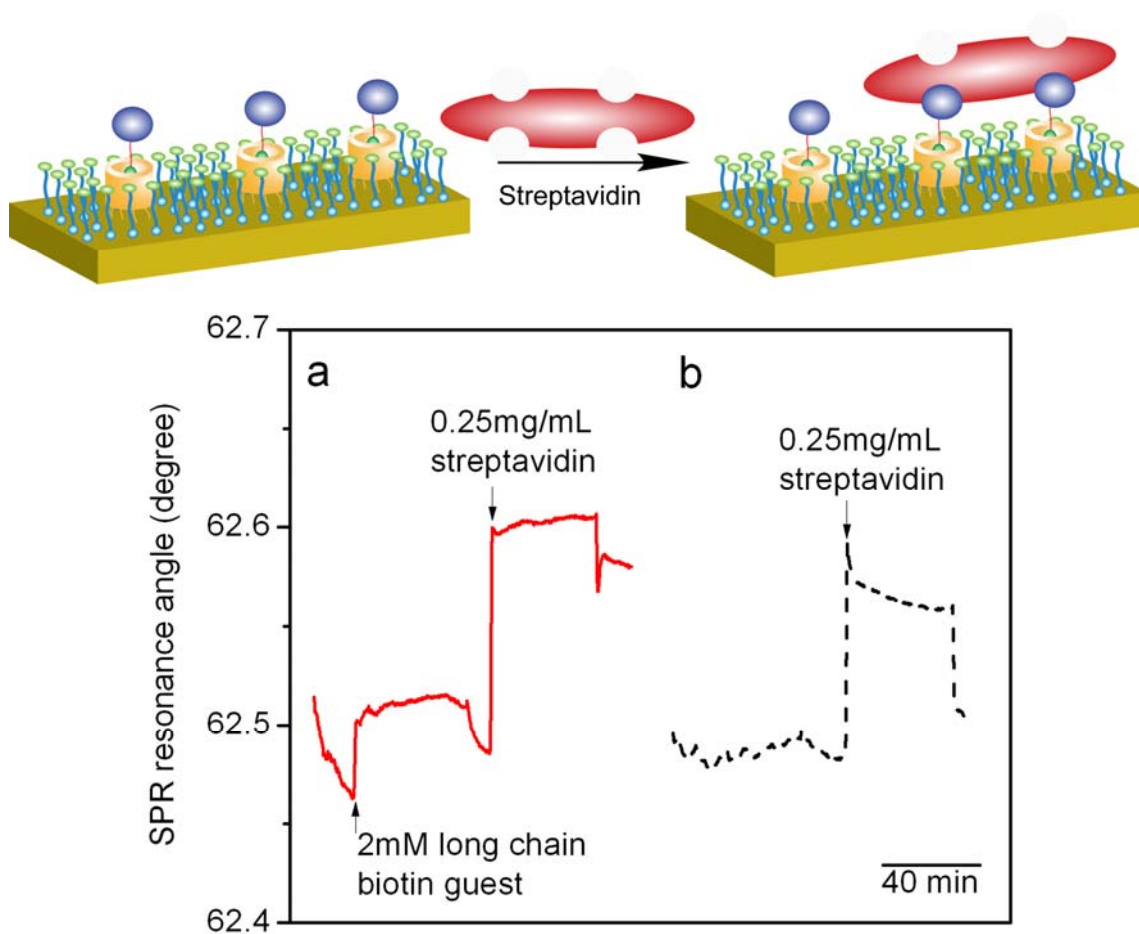


Figure 6.6. SPR sensorgram of streptavidin incubated with 10:1 MUA:Cavitand **3** SAM Au substrate a) in the presence and b) absence of biotinylated guest **5**.

was observed (Figure 6.6). Subsequent addition of a 0.25 mg/mL solution of streptavidin caused an increase in resonance angle of 0.14° , indicating the immobilization of the streptavidin protein at the surface. In the absence of cavitand (i.e. the MUA monolayer alone), minimal signal increase was observed, indicating that neither the guest nor the protein can be immobilized by MUA alone, and the biosensing process requires the specific non-covalent interaction of the cavitand: biotin guest: streptavidin complex.

6.4 Conclusions

This work has shown the first use of a *self-folding* deep cavitand on a gold surface. By incorporating sulfide feet on an octamide deep cavitand, surface attachment is possible. The attachment process can be studied by electrochemical and SPR methods. Complete monolayer formation is not observed from protic solvents due to the conformational flexibility of the host, however this can be circumvented either by performing the adsorption experiment in the presence of a template such as choline chloride that provides rigidity to the cavitand through host:guest interactions, or by addition of space-filling thiols to cover any gaps in the cavitand adsorption layer. The cavitand is capable of binding trimethylammonium-tagged guests from an aqueous medium, and the binding event can be monitored by surface plasmon resonance techniques. While the guest must protrude into the solvent for adequate detection, groups as small as a benzene ring can be detected by this method. The host can be deposited in 2 x 2 microarrays on the surface, and the binding of suitable guests allows detection of these arrays by SPR imaging techniques. If biotin-labeled guests are used, the cavitand:guest construct can

recognize and immobilize streptavidin proteins from aqueous solution, acting as a fully synthetic biosensor. Further work on the biosensing properties and synthesis of larger nanoconstructs is underway in our laboratory.

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Chapter 7 A Membrane-Bound Synthetic Receptor Promotes Growth of a Polymeric Archipelago at the Bilayer-Water Interface

7.1 Introduction

The central feature of eukaryotic cell structure is the presence of an outer polysaccharide cell wall surrounding a fluid membrane¹. This polymeric cell wall provides protection to the sensitive membrane structure, as well as incorporating molecular recognition motifs for cell signaling, transport and adhesion processes^{2,3}. Synthetic efforts to attach a polymer coating on a bilayer surface have been limited to the addition of preformed polysaccharides⁴ or covalent attachment of long PEG chains⁵, but these methods are limited in scope. An alternative strategy would be to grow a polymeric coating atop a biomimetic supported lipid bilayer, but this has been limited by the poor tolerance of the lipid bilayer to polymer growth conditions. Recent advances such as atom transfer radical polymerization (ATRP) are tolerant to aqueous media and can be performed under very mild conditions⁶. Indeed, polymer surfaces grown *via* ATRP are used as cushions to allow assembly of supported lipid bilayers on surfaces⁷. What is unprecedented, however, is to decorate the *exterior* of an established lipid bilayer with desired polymer patches. The obvious challenge in this approach is controlling the *attachment* of the outer polymer coating to the fluid membrane, as opposed to merely growing a polymer in and around the bilayer. Most studies of artificial membrane

constructs employ the covalent attachment of the desired motif to a lipid or steroid derivative that is incorporated in a synthetic membrane^{8,9}. To grow a polymer coating at the water-bilayer interface, the polymerization initiator must be incorporated in the bilayer itself. Creation of synthetic species that are capable of both selective membrane incorporation *and* reaction promotion or catalysis is challenging. To prevent the destruction of the lipid bilayer, the reaction conditions must be mild, and also the reactive species must be tolerant to the buffered aqueous conditions necessary for bilayer formation. Most examples of reactions at membrane surfaces consist of “bio-orthogonal” processes¹⁰ such as various azide-alkyne “click” cycloadditions¹¹⁻¹⁴, oxime formation¹⁵ and the thiol-ene reaction¹⁶, where the targets are covalently linked to steroid or lipid anchors and the reactive units are insensitive to the background media.

These efforts, while highly effective for robust bioconjugation, limit the scope of reactions that can be studied at the membrane surface. Recently, we introduced a different method of displaying functionality at a membrane bilayer surface; the incorporation of a water-soluble deep cavitand that can recognize trimethylammonium-tagged substrates and display the substrate to the exterior milieu, allowing immobilization of a number of species (including proteins) at the membrane surface¹⁷. Here we use this system to

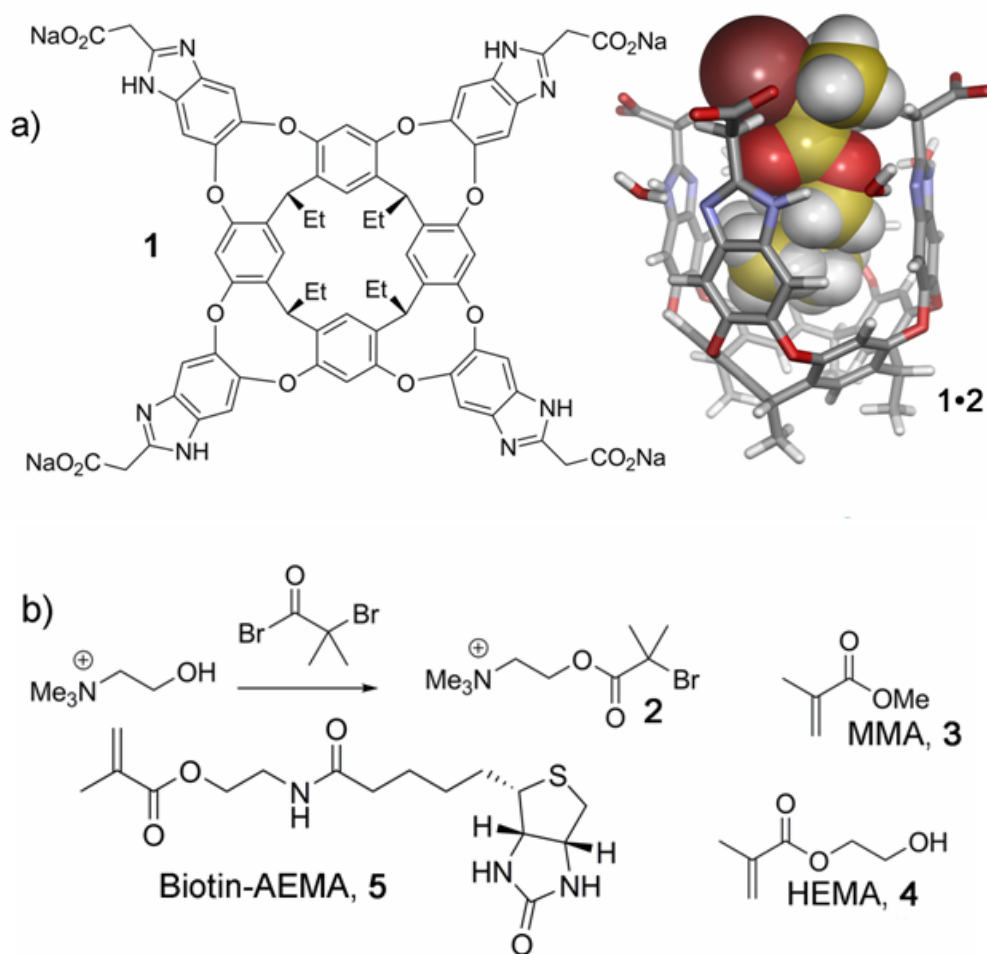


Figure 7.1. a) Tetracarboxylate cavitand **1** and the minimized conformation of **1** (SPARTAN, AM1 forcefield) with one bound initiator molecule (**2**) in the cavity; b) the guests and monomers used in this study; and c) representation of the polymer synthesis and regeneration experiment.

display a *reactive* initiator for atom transfer radical polymerization (ATRP)⁶ for the synthesis and installation of a functionalized polymeric archipelago at a supported lipid bilayer (SLB) interface through selective molecular recognition.

Tetracarboxylate cavitand **1** (Figure 7.1) is a water-soluble synthetic receptor that recognizes suitably sized hydrophobic species and biologically relevant molecules such as choline and other related trimethylammonium salts¹⁸⁻²⁰. The cavitand can be incorporated in either lipid-based micelles²¹⁻²⁴ or supported lipid bilayers¹⁷, while retaining its recognition properties for NMe_3^+ -tagged targets by exploiting cation- π interactions between the faces of aromatic cavitand walls and the charged guest. A significant advantage of cavitands as hosts is their open-ended character: long guests can extend out of the cavity, presenting large functional groups into the bulk solvent. For example, the display of biotin conjugates allows immobilization of avidin proteins at the surface¹⁷. The incorporation of cavitand and bound guests in the system is extremely mild; injected substrates are only exposed to water for 5-10 min. With this in mind, we explored the incorporation of a reactive initiator species **2**. Bromoester **2** is synthesized by combination of choline chloride with commercially available α -bromoisobutyryl bromide, and is capable of initiating atom transfer radical polymerization of methacrylate monomers under mild conditions in aqueous solution^{5, 7, 25}. Polymer coating has been previously used to form polymer cushions to display SLBs on surfaces^{26,27}. As it

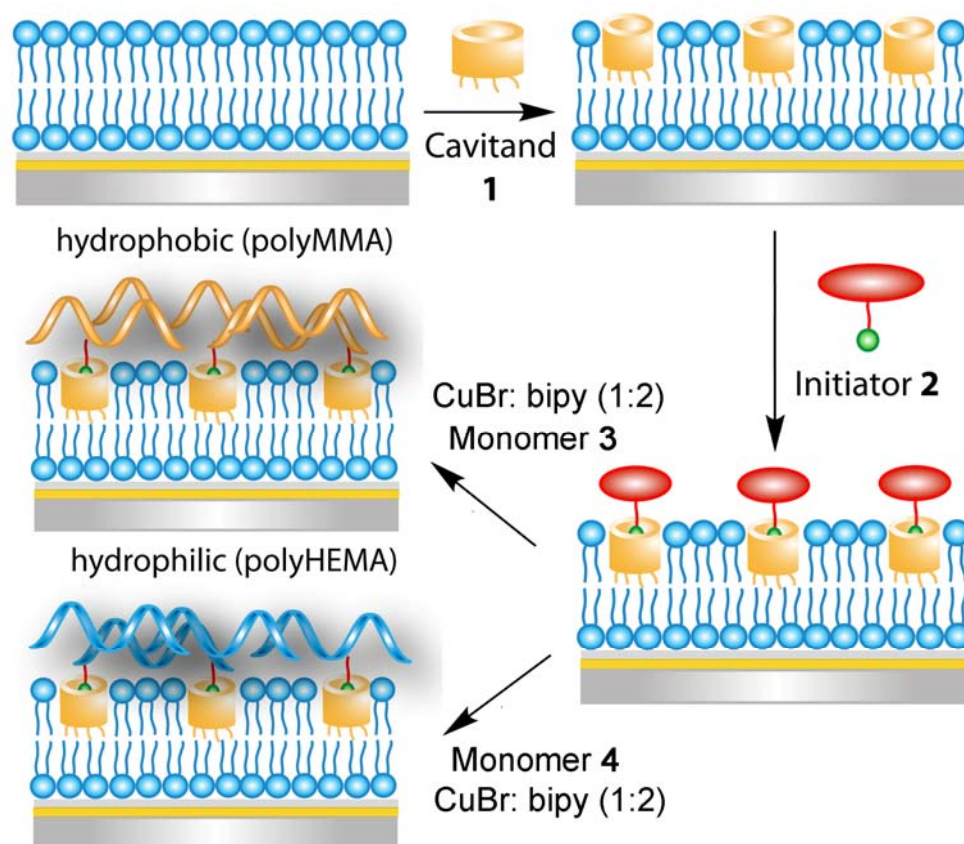


Figure 7.2. Representation of polymer growth at a cavitand-impregnated lipid bilayer.

demonstrated in Figure 7.2, by displaying the initiator at the membrane surface, it is possible to create polymers that are non-covalently attached to the *top* of the bilayer and form floating islands or archipelagos. The bilayer was fabricated by the injection of preformed L- α phosphatidylcholine (PC) vesicles that fuse readily on this chip. Sequential addition of an aqueous solution of cavitand **1** (0.8 mg/mL, 10% DMSO-water solution) followed by radical initiator **2** (10 mg/mL aqueous solution) *via* flowcell injection allows incorporation of materials.

7.2 Experimental

7.2.1 Instrument

^1H spectra were recorded on a Varian Inova 400 spectrometer. Proton (^1H) chemical shifts are reported in parts per million (δ) with respect to tetramethylsilane (TMS, $\delta=0$), and referenced internally with respect to the protio solvent impurity. Deuterated NMR solvents were obtained from Cambridge Isotope Laboratories, Inc., Andover, MA, and used without further purification. 1-Palmitoyl-2-oleoyl-sn-glycero -3-phosphocholine was purchased from Avanti Polar Lipids. Fluorescein conjugated avidin was purchased from Molecular Probes, Eugene, OR. All other materials were obtained from Aldrich Chemical Company, St. Louis, MO and were used as received.

7.2.2 Compound synthesis

2-(N,N,N-Trimethylammonium Bromide)-ethyl- α -bromoisobutyrate 2

Choline chloride (300 mg, 2.14 mmol) was added to CH₃CN (10 mL), followed by purging the system with N₂. α -Bromoisobutyryl bromide (540 μ L, 4.37 mmol) was added, and reaction was heated in the dark at 80°C. After 24 hours the reaction was allowed to cool, and then Et₂O (290 mL) was added to give a precipitate. After filtration and rinsing with additional Et₂O (50 mL), product was observed as a white solid (281 mg, 39%).

¹H NMR (400 MHz; D₂O) δ 3.64 (m, 2H), 3.24 (m, 2H), 3.15 (s, 9H), 2.31 (s, 3H). ¹³C NMR (100 MHz; D₂O) δ 173.0, 64.9, 60.4, 56.5, 54.5, 30.2. (ESI) m/z calcd for C₉H₁₉BrNO₂ (M⁺) 252.0593, found 252.0594.

2-(Biotinamidyl)ethylmethacrylate 5

Biotin (200 mg, 0.82 mmol) and 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU, 365 mg, 0.88 mmol) were added to MeCN (10 mL) along with Et₃N (400 μ L). After stirring for 1 hour, 2-aminoethylmethacrylate hydrochloride (136 mg, 0.82 mmol) was added to the reaction mixture along with additional Et₃N (400 μ L), followed by stirring in the dark. After stirring for 24 h the reaction mixture was evaporated *in vacuo* to give a solid. This was sonicated in 1M HCl (20 mL) until powdered, followed by filtration. The resulting tan

solid was then added to 1.5M NaOH (40 mL), followed by extraction with CH₂Cl₂ (4 x 50 mL). The combined organic phases were dried using MgSO₄ then evaporated *in vacuo* to give a yellow solid (44 mg, 15%).

¹H NMR (400 MHz; CDCl₃) δ=6.36 (br, 1H), 6.25 (br, 1H), 6.12 (s, 1H), 5.60 (t, *J* = 1.5 Hz, 1H), 5.31 (br, 1H), 4.51 (dd, *J* = 7.4, 4.8 Hz, 1H), 4.31 (dd, *J* = 7.4, 4.5 Hz, 1H), 4.25 (m, 2H), 3.56 (m, 2H), 3.14 (m, 1H), 2.91 (dd, *J* = 12.8, 4.8 Hz, 1H), 2.72 (d, *J* = 12.8 Hz, 1H), 2.22 (t, *J* = 7.4 Hz, 2H), 1.94 (s, 3H), 1.68 (m, 4H), 1.43 (m, 2H). ¹³C NMR (100 MHz; CDCl₃) δ= 173.7, 167.7, 164.3, 136.1, 126.3, 63.6, 61.9, 60.3, 55.9, 40.7, 38.7, 36.1, 28.4, 28.2, 25.8, 18.4. (ESI) *m/z* calcd for C₁₆H₂₅N₃O₄S ([M+H]⁺) 356.1638, found 356.1654.

7.2.3 Calcinated chip preparation

Gold substrates were fabricated with a 2 nm thick chromium adhesion layer, followed by the deposition of a 46 nm thick gold layer via e-beam evaporation on cleaned glass slides. The nanoglassified layers were constructed on the surface based on a previous layer-by-layer protocol. Clean gold substrates were immersed in 10 mM 3-mercaptopropionic acid (MPA) ethanol solution overnight to form a self-assembly monolayer. After extensive rinsing with ethanol and nanopure water and drying with nitrogen gas, modified gold substrates were alternated dipped into sodium silicate solution (22 mg/mL, adjusted to pH 9.5) and poly(allylamine hydrochloride) solution

(1mg/mL, adjusted to pH 8.0) for 1min to form a layer by layer assembly structure, with sufficient ultrapure water rinsing between layers. This dipping process was repeated six times to build up a multilayer membranes gold chip, followed by calcinated in a furnace by heating to 450 °C at a rate of 17 °C per min and allowing cooling to room temperature 4 hours later.

7.2.4 Vesicle preparation

PC lipid stock solution was transferred to a small vial and the organic solvent was purged from the vial with N₂ to form a dry lipid film on the vial wall, which was then rehydrated with 20mM PBS (150mM NaCl, pH 7.40) to a lipid concentration of 1 mg/mL. The resuspended lipids were probe sonicated for 20 min, followed by centrifugation at 8000 rpm for 6 min to remove any titanium particles released from the probe tip. The supernatant was then extruded with 11 passes through a polycarbonate membrane of pore size 100 nm to ensure formation of small unilamellar vesicles. The solution was then incubated at 4 °C for at least 1 h before use.

7.2.5 Fabrication of cavitand 1 receptor layer, initiator binding and ATRP reaction measurement

The fabrication of cavitand **1** - membrane complex and subsequent guest binding was monitored by surface plasma resonance (SPR) spectrometry. The shift of SPR minimum angle characterizes surface refractive index change, demonstrating adsorption or binding

on the surface. The calcinated gold substrate was rinsed with ethanol and nanopure water. After dried under a gentle stream of N₂ gas, the gold substrate was clamped down by a flow cell on a high-refractive index prism for SPR measurement. 1 mg/mL PC vesicles in 20mM PBS (150mM NaCl, pH 7.40) were injected through a flow-injection system, and incubated 1 h to allow vesicle fusion on the hydrophilic calcinated gold surface, forming a smooth bilayer membrane. After 10 min rinsing to remove excess vesicle from the surface, 2 mg/mL cavitand **1** in 10% DMSO solution was subsequently injected and incubated for 20 min. The surface was extensively rinsed with water, followed by incubation with 10mg/mL initiator **2** aqueous solution for 30 min. After 10 min rinsing to remove unbound initiator **2** from bilayer lipid membrane, atom transfer radical polymerization (ATRP) reaction was initiated by the injection of monomer **3-5** (0.3M MMA **3**, 1.6M HEMA **4**, and 0.04M Biotin-AEMA **5**) and catalyst (CuBr/2,2'-bipyridine/L-ascorbic acid in a 1:2:1.5 molar ratio) mixture solution. After 30 min incubation for polymer growth, ATRP reaction was terminated by 10 min rinsing with water. PBS rinsing was followed to remove hydrophilic poly(HEMA).

After poly(Biotin-AEMA) was generated on lipid membrane surface, fluorescein conjugated avidin was injected and incubated for 30 min, followed by water rinsing to remove the uncaptured avidin.

7.2.6 FRAP Measurement

Fluorescence recovery after photobleaching (FRAP) experiments were performed by making fluorescent vesicles containing 5% NBD-PC and 95% PC. After vesicle preparation similar to that noted above, a cleaned glass slide was mounted on the SPR prism, and 100 μ L of vesicle solution was injected onto the glass slide with the flow injection system coupled with SPR. Vesicles were incubated on glass slide surface for 1 h to allow the vesicles to fuse to bilayer lipid membrane. ATRP reaction was triggered from lipid membrane surface with the same method noted above by injection of monomer 0.3M MMA and catalyst mixture solution and 30 min incubation for polymer growth. FRAP experiments were carried out on a Zeiss LSM 510 confocal laser scanning microscope (CLSM) with 488 nm argon laser excitation, a CCD camera, and a 527 nm long-pass emission filter with a 40x dipping objective. The objective was focused onto the bilayer lipid membrane fusion region. The scanning mode of the CLSM was used, and after three pristine surface images were taken, a vertical line was bleached with new images taken at 3s intervals with a CCD camera. Fluorescence recovery was monitored with time, and the data was fit to the model developed by Koppel⁷ to determine diffusion coefficients.

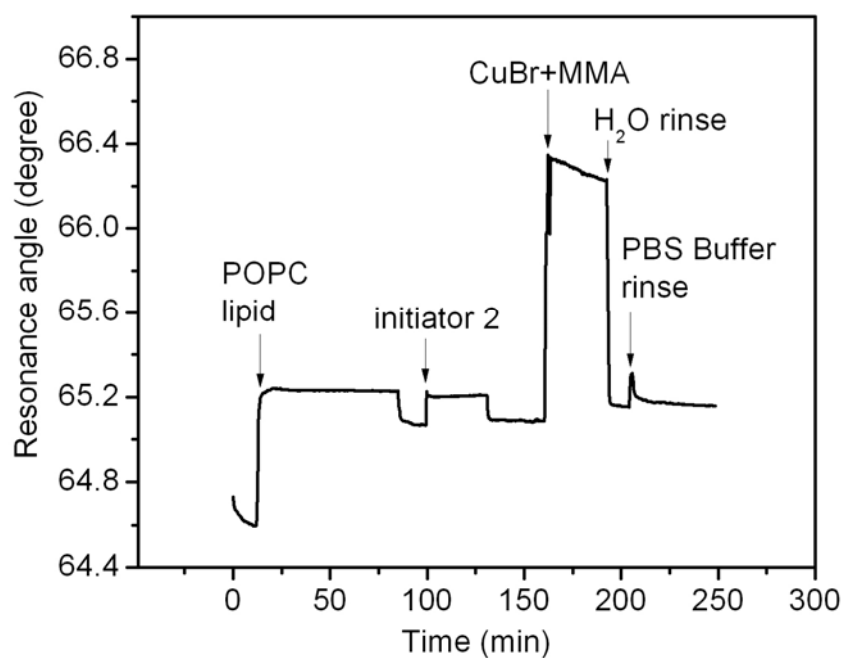
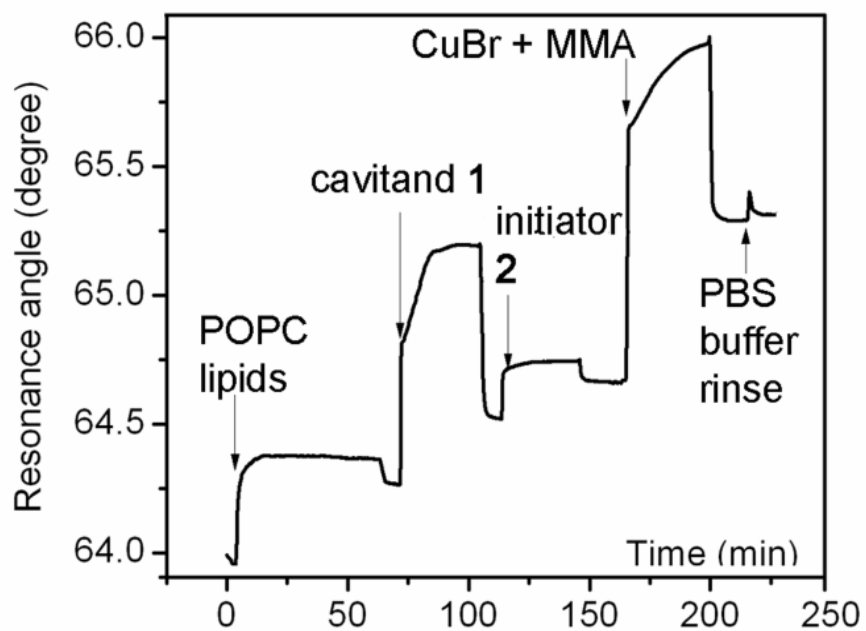


Figure 7.3. SPR sensorgram for cavitant-mediated synthesis of poly(MMA) (above) and control experiment in the absence of cavitant (bottom).

7.3 Results and discussion

7.3.1 SPR characterization of poly(MMA) grown from lipid membrane surface

The initial test monomer was methylmethacrylate **3** (MMA). After the injection of a 0.3 M aqueous solution of **3** in the presence of catalytic mixture of CuBr, 2,2'-bipyridyl (bipy) and ascorbic acid (in a 1:2:1.5 ratio) a slow increase in SPR signal was observed (Figure 7.3 above), corresponding to the formation of poly(MMA) at the membrane surface. After 20 min, the excess monomer was rinsed from the flowcell, and reaction (and concomitant SPR signal increase) ceased. The attached polymer was resistant to washings: the system was rinsed with both water and PBS solution, neither of which caused removal of the polymer from the system. Addition of choline chloride as a competitive guest for the initiator “anchors” did not remove the hydrophobic polymer from the surface, most likely due to inaccessibility of the cavitands to the exterior medium after polymerization has occurred. Control experiments were performed in the absence of the cavitand (Figure 7.3 bottom). No incorporation of initiator **2** into the membrane was observed, and no growth of polymer occurred upon addition of MMA and CuBr catalyst, although MMA monomer **3** caused a slight increase in SPR signal due to weak nonspecific interaction with the SLB. The living nature of the polymerization can

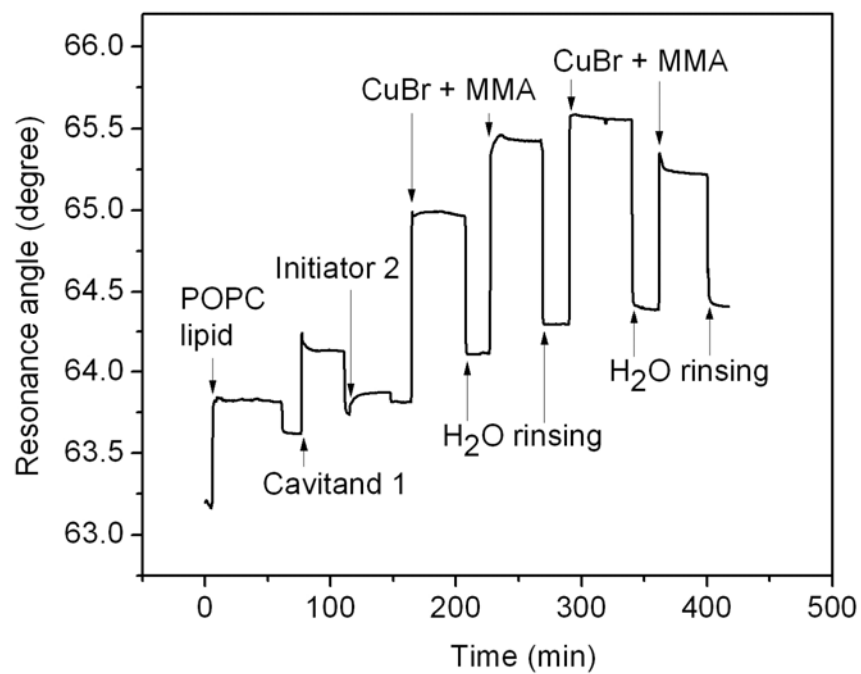


Figure 7.4. SPR sensorgram indicating the living polymer growth by repeating injection of monomer MMA and catalyst.

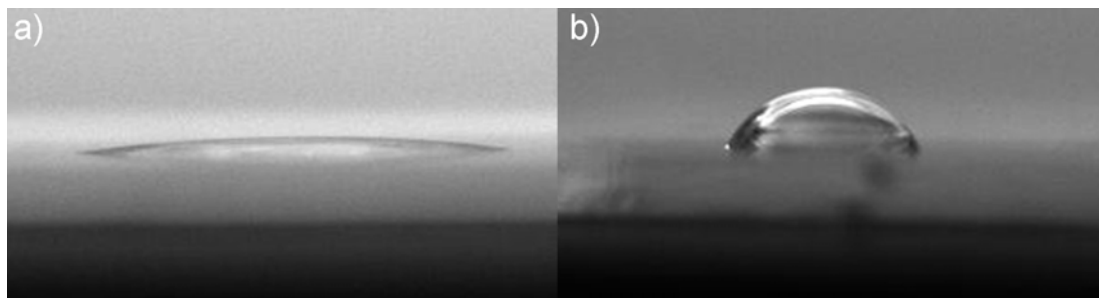


Figure 7.5. Effect of *in situ* polymerization on film hydrophobicity. a) POPC supported membrane; b) after growth of poly(MMA) at the cavitand-embedded membrane surface.

be tested by repeating polymer growth after excess monomer is removed by washing. Polymer growth can be restarted after removal of monomer by simple reinjection of monomer and catalyst mixture 4 times (Figure 7.4).

The SPR sensorgrams allow calculation of surface coverage upon adaptation of Jung's formula²⁸. The surface coverage of cavitand 1 was estimated to be 1.53×10^{-10} mol/cm². The surface coverage of freshly grown poly(MMA) was determined to be 1.58×10^{-8} mol/cm², with a calculated thickness of approximately 17 nm.

7.3.2 Contact angle measurement

Evidence for the attachment of the polymer to the bilayer surface is shown in Figure 7.4. The lipid membrane-covered chip is hydrophilic due to the charged headgroups of the phosphocholine lipids. Contact angle measurement shows a flattened water droplet spread out across the surface (Figure 7.5a) that displays a contact angle of 10.5°. After poly(MMA) formation, a fully formed drop is observed at the surface with a substantially increased contact angle of 53.9° (Figure 7.5b).

7.3.3 SPR characterization of poly(HEMA) grown from lipid membrane surface

The nature of the polymeric archipelago can be varied. When 2-hydroxyethyl methacrylate (HEMA) **4** was used as monomer, a *hydrophilic* polymer (poly(HEMA)) was formed at the membrane surface, and the process was again monitored by SPR (Figure 7.6). No loss of polymer was observed upon washing with water, but the

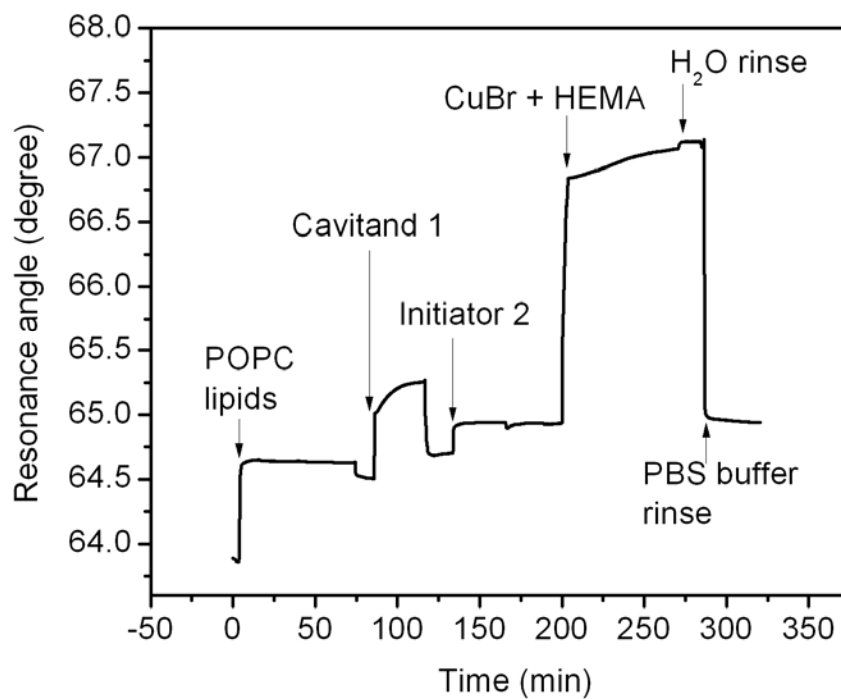


Figure 7.6. SPR sensorgram for cavitaand-mediated synthesis of poly(HEMA).

poly(HEMA) patch was significantly less intractable than the poly(MMA) in salt solution and could be removed from the bilayer interface by a simple wash with PBS buffer (Figure 7.6). Cavitand **1** is well-known to display lower binding affinities for its targets in high salt conditions ¹⁹ and it appears that even the multiple anchors from initiation are not sufficient to hold the hydrophilic poly(HEMA) at the bilayer surface under the shear forces applied in the flowcell, and the hydrophilic polymer can be selectively removed by this treatment.

This experiment allows analysis of the state of the membrane bilayer *after* polymerization, something not possible with the immovable poly(MMA). SPR analysis indicates that the supported lipid bilayer remains fully intact throughout the experiment. We therefore carried out additional polymer growth on the same membrane (Figure 7.7a) after washing with PBS buffer to remove the membrane-bound polymer. The first experiment was to determine whether the cavitand was still functional in the bilayer. Initiator **2** was injected into the freshly regenerated chip, followed by a HEMA **4**/CuBr solution. Polymer growth was observed again at the bilayer interface, indicating that the cavitands were not removed from the bilayer, and remained bound and functional in the membrane. Most importantly, this experiment also shows the presence of *initiator* molecules in the cavitands. Repeated poly(HEMA) growth did not require addition of further initiator molecules (Figure 7.7b). When HEMA **4** and CuBr were injected to the

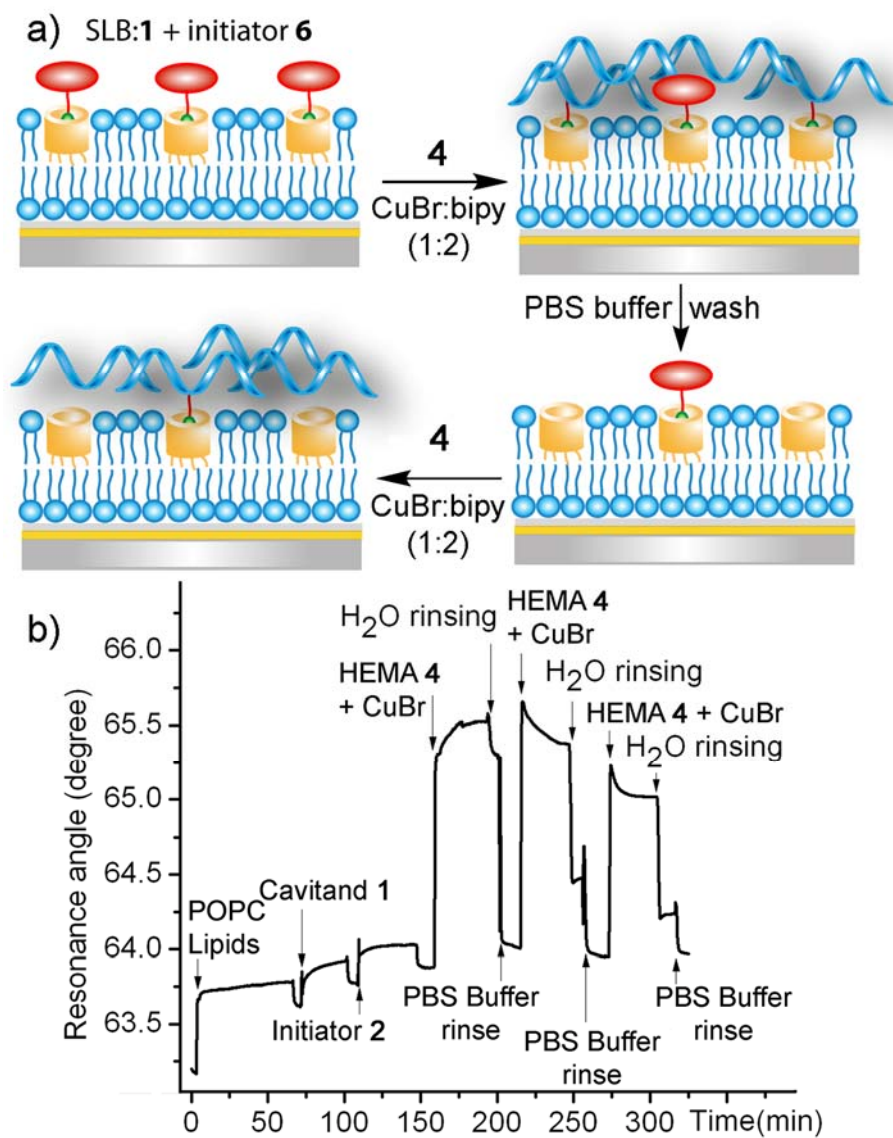


Figure 7.7. a) Scheme of reversible polymer growth and b) SPR sensorgram indicating the repeated removal/construction process with poly(HEMA).

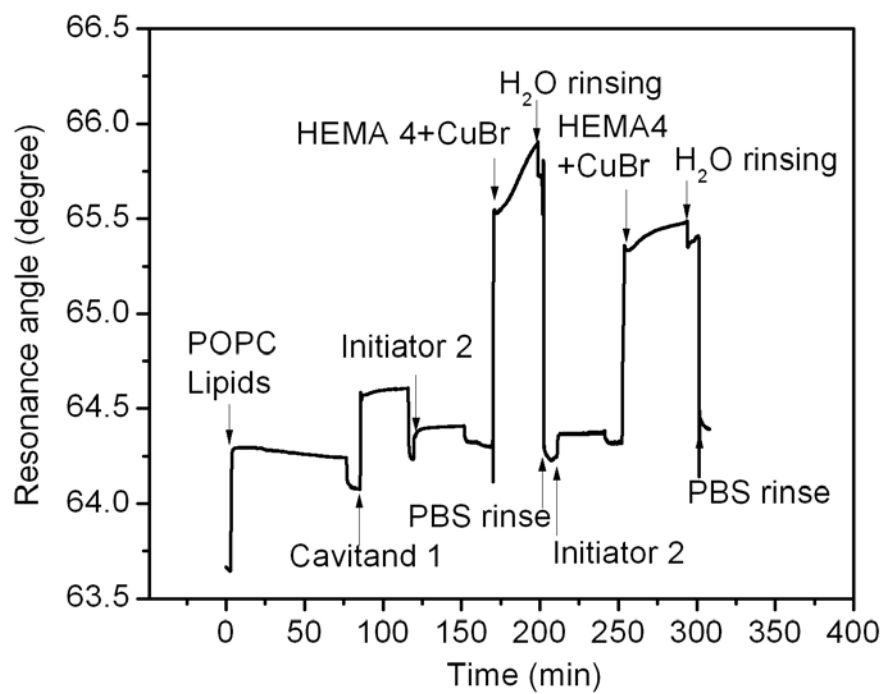


Figure 7.8. Repeat ATRP reaction with second injection initiator.

system without new addition of initiators, growth of poly(HEMA) could be observed, albeit to a smaller extent. The polymer size can be tracked by SPR measurements: after the initial reaction (and *aqueous* wash), an increase in resonance angle of 1.37 degrees was observed. This increase was lessened to 0.47 degrees for the second growth after poly(HEMA) removal, and to 0.27 degrees after the third experiment. Evidently not all bound initiator molecules are used up in the initial polymerization, and remain intact, bound in the cavitand hosts underneath the growing polymer. Upon washing, the “used” initiators are removed from the cavitand along with the polymer, leaving fresh initiator at the membrane surface, suitable for future reaction. If a new batch of initiators was added to the system after each washing, the polymer growth occurs at its maximal levels. The unused cavitands are ready for “refilling” with new reactants (Figure 7.8).

7.3.4 Functional polymer grown from lipid membrane surface

Functional polymers can also be created at the membrane interface. If Biotin-AEMA monomer **5** is introduced to the system, a bioadhesive polymer can be formed. Biotin-AEMA **5** was synthesized by reaction of biotin with 2-aminoethylmethacrylate hydrochloride (AEMA), using HCTU as the coupling agent. Monomer **5** is only sparingly water-soluble, and was introduced to the bilayer:**1:2** complex with catalytic CuBr as before in a 10:1 water:DMSO solution. The biotin-containing **5** is a suitable ATRP monomer, and polymer growth proceeded smoothly upon exposure of the catalyst and

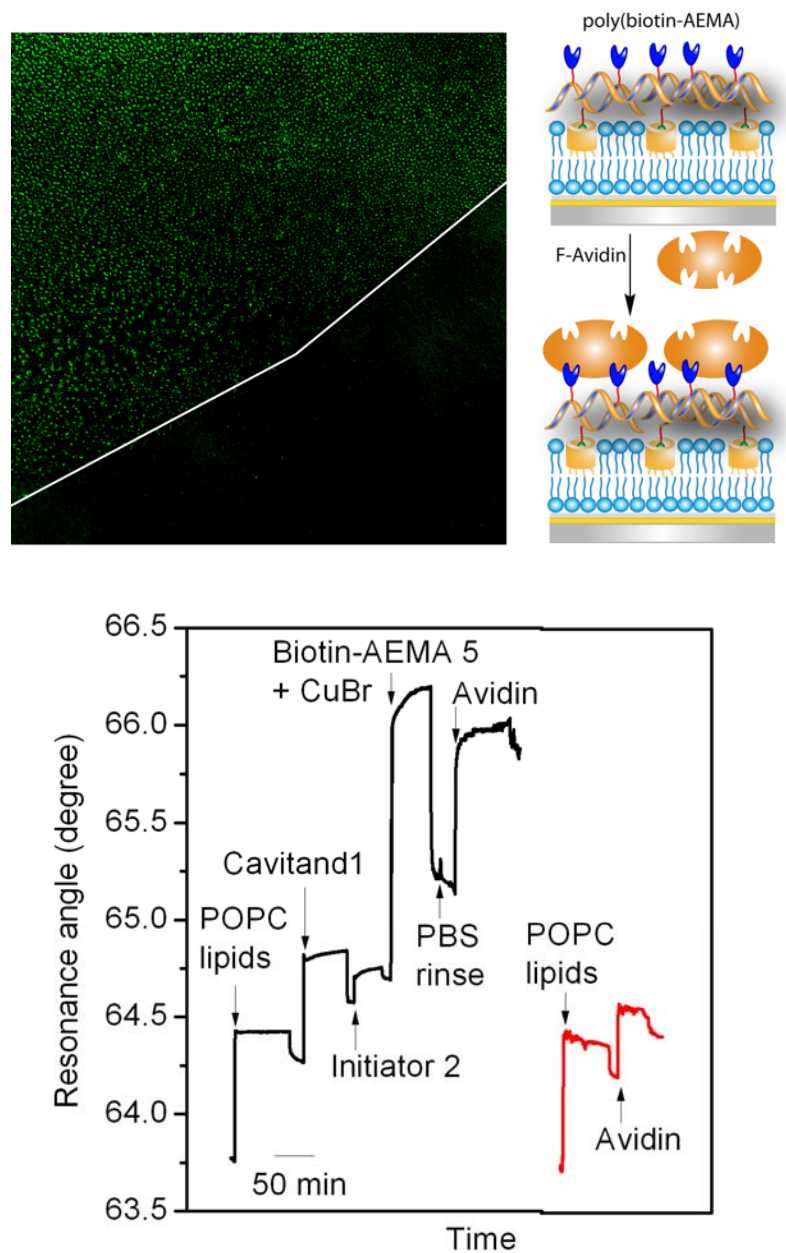


Figure 7.9. Fluorescence microscopy image of fluorescently tagged avidin bound to a poly(biotin-AEMA) polymer grown at the membrane interface and cartoon of the experiment (top). SPR sensorgram demonstrating avidin-fluorescein attached on poly(biotin-AEMA).

monomer to the membrane-bound initiator:cavitand complex, as monitored by SPR. Poly(Biotin-AEMA) was resistant to removal from the surface by PBS washing, indicating its relative insolubility in water. The poly(Biotin-AEMA) is a bioadhesive surface, and treatment of the membrane-bound polymer with avidin allows capture of the protein at the polymer surface. The adhesion process could be monitored by confocal fluorescence microscopy. The microscopy image of the adhesion is shown in Figure 7.9 above, and illustrates the immobilization of the avidin-fluorescein conjugate by the bioadhesive polymer. The white line delineates the edge of the chip, showing no protein adhesion in the absence of the bioadhesive polymer. SPR analysis was also carried out using fluorescently-tagged avidin, after the injection and incubation of 0.25 mg/mL avidin-fluorescein conjugate to the preformed polymer-coated membrane, a change in resonance angle of 0.71° was observed (Figure 7.9 bottom). On plain POPC membrane, there was a slight nonspecific retention of the avidin-fluorescein conjugate, but the observed resonance angle change was minimal.

7.3.5 FRAP testing of the mobility of lipid membrane before and after polymer growth

FRAP has been proven useful to test lipid membrane mobility because lateral mobility in a membrane is necessary for intrinsic biological functionality and the results can enable the determination of the diffusion coefficients, D . FRAP experiment were

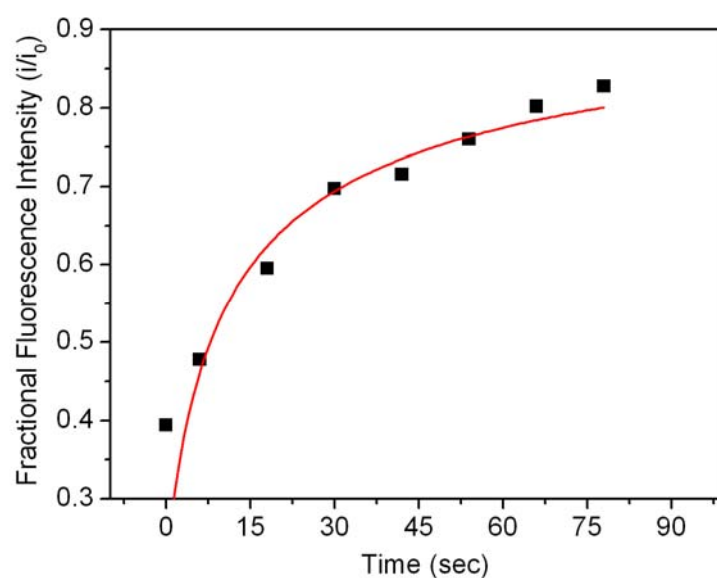
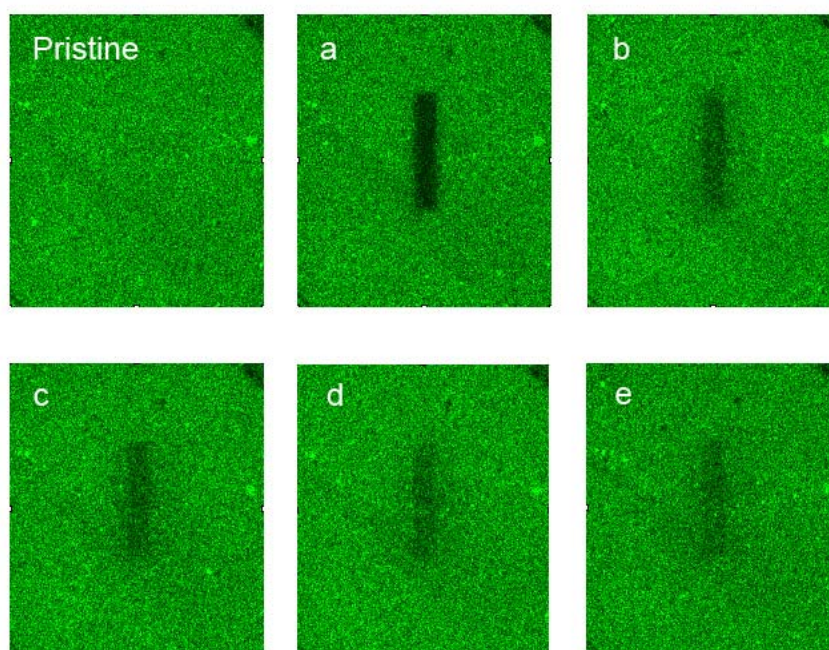


Figure 7.10. Confocal microscopy images of the FRAP experiment for a supported lipid bilayer consisting of 5% NBD-labelled PC and 95% PC lipids on a cleaned glass slide. Pristine refers to membrane before photobleaching, and a-e show images obtained 3, 21, 39, 57 and 75 s after bleaching, respectively (above). FRAP recovery fitting curve obtained with a supported lipid bilayer consisting of 5% NBD-labelled PC and 95% PC lipids on a cleaned glass slide (bottom).

conducted on the bilayer lipid membrane from calcinated substrates before and after poly(MMA) growth to demonstrate the mobility change. Figure 7.10 is a representative example of FRAP results with fluorescent time-stamped images (above) and a recovery curve (bottom). The diffusion coefficient is determined as $3.98 \pm 0.85 \mu\text{m}^2/\text{s}$, which agree well with previously studied PC-based membranes on glass where diffusion coefficients generally ranged from 1 to $4 \mu\text{m}^2/\text{s}$ ²⁹. For the polymer coated bilayer membrane, however, the fluorescence intensity was considerably lower, and the diffusion coefficient was reduced to $0.09 \pm 0.02 \mu\text{m}^2/\text{s}$. This indicates that the presence of the polymeric archipelago substantially rigidifies the membrane bilayer underneath it, limiting the lipid movement and thus lowering the lateral mobility as reflected by the diffusion coefficient.

7.4 Conclusion

In summary, we have shown that a membrane-bound water-soluble deep cavitand can be used to bind an ATRP initiator that can initiate polymer growth at the membrane-water interface of a supported lipid bilayer. Polymer growth only occurs in the presence of cavitand, as the initiator molecule is not incorporated in the membrane itself. Both hydrophobic and hydrophilic polymers, as well as bioadhesive polymers can be synthesized by simple alteration of the monomer. Polymer synthesis can be observed in real time by SPR analysis and in the case of hydrophilic polymer poly(HEMA), can be repeated several times after washing. The supported lipid bilayer remains intact

throughout the reaction, and both the cavitands and unused initiator molecules remain incorporated in the supported lipid bilayer throughout the process. The mild experimental condition and anchored polymer growth provide crucial breakthrough for installation of functional patches on a fluid lipid membrane. It opens new avenues for investigation of cell-membrane adhesion properties on a substrate surface using spectroscopic and microscopic approaches. Further studies on the properties of host molecules as membrane-bound anchors for nanoconstruction and biosensing are underway in our laboratories.

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Chapter 8 SPR/MALDI-MS Analysis of Proteins and Peptides on a Thermoresponsive Polymer-Coated Au Substrate

8.1 Introduction

The genome, proteome, and glycome of an organism constitute a mixture of several millions of molecular species involved in an intermolecular network. There is an increasing demand for methods that provide kinetic and thermodynamic characterization of the biomolecular interactions, as well as quantification and identification of biomolecular partners through their selective extraction and enrichment from cellular compartments¹. Therefore, coupling of quantitative macromolecular analysis techniques such as surface plasmon resonance (SPR) to downstream detection methods in particular mass spectrometry, has attracted considerable interest in the search of effective approaches². SPR is able to register the total amount of biomaterial retained on the surface and determine reaction rate constants and equilibrium constants. The SPR detection itself is nondestructive to the proteins being analyzed, the same proteins that are affinity-retrieved on the SPR chips can be further analyzed via mass spectrometry. Matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) has become one of the most important tools for peptide and protein analysis in proteome research due to its high ionization efficiency, high sensitivity, rapidity, capability of high throughput,

and ease of automation³. In combination with SPR, MS and tandem MS/MS can be used to identify the specifically retained ligands through the measurement of their molecular weights and fragmentation patterns^{4, 5}.

Coupling between SPR devices and mass spectrometers has been investigated for some time^{6, 7}. To develop the approach to couple SPR and MS requires specific practice for successful operation. One approach is based on the recovery by elution from the biochip surface and their subsequent MS analysis. In this protocol, a capture protein or peptide is immobilized on a sensor chip. Complex biological solution is injected through the chip into the fluidic system. Noninteracting proteins from the biological sample are then washed away, and proteins that have bound to the immobilized ligand are recovered by injection of the elution buffer. These interacting proteins are then recovered and digested into peptides that are separated and analyzed with MS system. The sensor chip with immobilized protein or peptide can then be used for various recovery experiments⁸. The eluted ligands obtained by the microrecovery procedure can be analyzed using the MALDI⁹, surface-enhanced laser desorption ionization (SELDI)², or electrospray ionization (ESI)¹⁰ ion sources. This strategy is broadly employed because most of the SPR-MS coupling devices described in literature operate with a flow cell. However, the recovery procedure is known to be time-consuming and leads to material loss and contamination, while the quantitative elution could be challenging in the case of a

high-affinity interaction. Another approach consists of direct on-chip MS analysis¹. Considering that the SPR surface could be easily interfaced with the mass spectrometer, the direct on-chip MS analysis may overcome the above mentioned limitations, and is appropriate for high throughput analysis. MALDI appears as the most versatile method for on-chip MS analysis¹¹.

MALDI experiment is known to depend on the co-crystallization condition for matrix-analyte mixtures on the sample plate. The existence of contaminants such as salts often impedes the crystallization process, resulting in significant decrease of sensitivity and reproducibility in MS detection. However, high salt buffers must be used in biological samples to stabilize the molecules and maintain their activities, which made sample desalting a crucial step in MALDI-MS analysis of biomolecules including peptides and proteins. Miniaturized sample preparation techniques, such as micro- or nanoscale solid phase extraction (SPE), have been developed to reduce the sample size and simplify the sample handling process^{12, 13}. An effective approach is to conduct SPE in pipette tips packed with chromatographic materials such as ZipTip (Millipore, Bedford, MA), HyperSep (ThermoScientific, Rockford, IL) and StageTips (Proxeon, Cambridge, MA)¹³. However, the off-line tip desalting method can not be applied to direct on-chip MALDI-MS analysis after SPR detection.

Controlling the surface hydrophobicity is the most effective method for on plate concentration and desalting. A number of methods have been reported to increase surface hydrophobicity, including Teflon¹⁴ and paraffin wax film¹⁵ coating, radiate microstructure¹⁶, self-assembled monolayers (SAM)¹⁷, and octadecyltrichlorosilane (OTS) modified porous silicate nanofilm generated by layer by layer deposition and calcinations^{13, 15}. Hydrophobic polymer grafted surface have been used for MALDI analysis, including poly(methyl methacrylate) materials^{18, 19}, polyethylene, and polypropylene²⁰. Polycation (polyethylenimine or poly(acrylic acid) complexed with Fe^{3+}) grafted surface can selectively bind the phosphorylated peptides and showed decontamination ability²¹. Block copolymer polysulfone-poly(ethylene oxide) is able to embed salts with its hydrophilic domain of poly(ethylene oxide), and concentrate peptides with its hydrophobic domain of polysulfone²².

Stimuli-responsive polymer graft can generate thermally, pH, or optically responsive smart interface with tunable wettability and reversible switching between hydrophilicity and hydrophobicity. Poly(N-isopropylacrylamide) (PNIPAAm) shows a lower critical solution temperature (LCST) of 31° in an aqueous environment, thus the hydrophobicity of PNIPAAm grafted surface can be switched on and off by changing temperature. Below the LCST, the PNIPAAm grafted surface is hydrophilic and protein nonadsorptive. As the temperature increases above the LCST, the grafted polymer chain collapse and the

surface becomes hydrophobic and protein retentive. A dual-responsive surface to both temperature and pH was fabricated by generating a poly(N-isopropyl acrylamide-co-acrylic acid) thin film on silicon substrate, which can be switched between superhydrophilicity and superhydrophobicity²³. 70% PNIPAAm and 30% polymethacrylic acid (PMAA) was synthesized on gold substrate and applied in separating cationic peptide bradykinin and anionic peptide buccalin for MALDI-MS analysis²⁴. Plasma PNIPAAm polymer modified MALDI probe has been used for protein on-probe purification²⁵, but the MALDI-MS result is not satisfactory due to the limited surface density.

We report here an on-plate desalting method on PNIPAAm modified substrate, and coupled SPR with MALDI-MS for quantitative detection and recognition of binding peptides. The method is based on PNIPAAm hydrophilicity / hydrophobicity change upon temperature switch. By converting the surface self-assembly monolayer hydroxyl group to initiator²⁶, surface initiator atom transfer radical polymerization (SI-ATRP) reaction was triggered on the gold surface to generate PNIPAAm polymer chain, which is then manipulated to retain target analytes on surface while major contaminants including salts and contaminants are selectively removed by hot water rinsing. SPR was applied to characterize *in-situ* polymer configuration change and monitor peptide binding. Matrix was added at room temperature; the retained peptides were released by hydrophilic

PNIPAAm polymer chain and evenly co-crystallized with matrix. MALDI-MS was performed on the gold substrate where matrix deposited. The coupling method demonstrates quantitative detection of 120 pmol neurotensin peptide, and the peptide peak is well recognized from mass spectra. PNIPAAm on-plate desalting has also been successfully applied to analysis of peptides in α -casein protein digests, and provided comparable result to commercial C18 desalting tip.

8.2 Experimental

8.2.1 Materials

6-mercapto-1-hexanol (MCH), 2-bromoisobutryl bromide (BIBB), triethylamine (TEA), dimethylformamide (DMF), N-isopropylacrylamide (NIPAAm), CuBr, 2,2'-bipyridyl, L-ascorbic acid (AA), cytochrome c from bovine heart, neurotensin (MW = 1672), α -casein ($\geq 70\%$, from bovine milk), trypsin (from bovine pancreas), sodium bicarbonate, and ammonium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO). Ethanol, acetonitrile (ACN), trifluoroacetic acid (TFA), α -cyano-3-hydroxycinnamic acid (CHCA) were purchased from Thermo-Fisher Scientific (Pittsburgh, PA). C18 reversed phase HyperSepTM desalting tip was purchased from ThermoScientific (Rockford, IL). Protein solutions were prepared in 20 mM phosphate buffered saline (containing 150 mM NaCl, pH 7.4). Water was purified by a Milli-Q system. All other reagents were analytical grade and used without further purification.

8.2.2 Preparation of poly(N-isopropylacrylamide) (PNIPAAm) grafted substrate and SPR characterization

Surface modification with PNIPAAm polymer graft and protein/peptide adsorption were monitored using surface plasmon resonance (SPR) spectrometer NanoSPR-321 (NanoSPR, Addison, IL) with a GaAs semiconductor laser light source ($\lambda = 670$ nm). The device comes with a high-refractive index prism ($n = 1.61$) and 30 μ L flow cell. A temperature control device is coupled to the flow cell to adjust the temperature between room temperature and 60 °C. SPR gold substrates were fabricated with a 2 nm thick chromium adhesion layer, followed by deposition of a 46 nm thick gold layer via e-beam evaporation onto cleaned BK-7 glass slides.

After immersed in a piranha solution (7:3 v/v, $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$) (Caution!) for 1 min to remove inorganic and organic contaminants on the surface, the gold substrate was incubated in 1mM MCH ethanol solution overnight to form a self-assembled hydroxyl groups on the surface. After extensive rinsing with copious ethanol and DI water, the chip was dried under a N_2 stream. Direct coupling of 2-bromoisobutyryl bromide to the surface hydroxyl groups was achieved by immersing in a DMF solution of 0.08 M BIBB and 0.1M TEA for 20 minutes, followed by DI water rinsing and N_2 stream dry. The gold substrate was then clamped to a flow cell on the SPR prism. The atom transfer radical polymerization (ATRP) polymer growth was triggered by the injection of an aqueous

solution of 2 M NIPAAM monomer and catalyst (9 mM CuBr / 18 mM 2,2'-bipyridine / 13.5 mM AA) (Figure 8-1). AA was added as oxygen suppressor to prevent catalyst from deactivation²⁷. The monomer and catalyst solution was purged with nitrogen for 30 min before injection to reduce the amount of O₂ present. After incubation for 30 min, the polymerization was stopped by removal of the NIPAAM / catalyst mixture with PBS buffer. ATRP reaction was conducted at room temperature and high temperature respectively. For the experiment conducted at high temperature, temperature was adjusted to 60 °C by the temperature control device coupled to SPR.

The binding assay was carried out with injection of 1 mg/mL cytochrome c and 1 mg/mL neurotensin respectively. Cytochrome c was incubated on surface for 30 min before 10 min buffer rinsing. 1 hour incubation for neurotensin was performed to ensure sufficient binding of smaller molecular mass peptide.

8.2.3 Characterization of PNIPAM grafted surface

Contact angle measurements were performed on a home-built device with deionized water (2 µL) at room temperature and 60°C. The images for water droplets on substrate were collected by a computer controlled 12-bit cooled CCD camera.

8.2.4 Sample preparation

α -Casein (1 mg/mL, 1 mL) was mixed with trypsin (0.2 mg/mL, 100 μ L) in aqueous ammonium bicarbonate (50 mM, pH 8) and incubated at 37 °C for 24 h. The reaction was stopped by adding 2% TFA solution.

The desalting of digested peptide mixture was performed by commercial C18-HyperSep desalting tip. The C18 desalting tip was attached to a micropipette and aspirated/expelled 20 μ L 50% (v/v) acetonitrile containing 0.1% TFA aqueous solution 5 times followed by 3 times aspirate/expel of 0.05% TFA solution for tip conditioning. 10 μ L digested peptide mixture sample was aspirated/expelled by the prepared tip 50 times for sample binding to allow the peptides to adsorb to the reversed phase material of desalting tip. Sample washing was conducted by 10 times aspirated/expelled 20 μ L 0.05% TFA solution, the expelled solution was discarded each time. In the following, the adsorbed sample was released from desalting tip by 10 times aspirated/expelled of 10 μ L 50% (v/v) acetonitrile containing 0.1% TFA aqueous solution, the expelled solution was collected in a suitable clean tube and stored at -20°C until further use. The desalting peptide mixture was directly diluted 200 times with 50% (v/v) acetonitrile containing 0.1% TFA aqueous solution before further analysis.

8.2.5 On-plate desalting and enrichment of neurotensin and peptide digest sample

As shown in Figure 8.1, 5 μ L of neurotensin or α -Casein digests were pipetted to PNIPAAm grafted surface and incubated for a certain time in a homemade humid chamber at room temperature. The sample spots and the adjacent area were then rinsed intensively by 60°C DI water to remove salts, contaminants, and surfactants. Finally, 1 μ L of matrix CHCA aqueous solution (10 mg/mL, 50% acetonitrile, and 0.1% TFA) was pipetted on protein spots at room temperature and leave in humid chamber at room temperature for solvent evaporation and cocrystallization. A homemade sample stage was used to hold the substrate for MALDI-MS analysis. MALDI-MS was directly performed on matrix deposited area.

8.2.6 MALDI-TOF MS

Matrix-assisted laser desorption / ionization mass spectra were obtained by using a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA). Experiments were performed in positive reflector mode at accelerating voltage of 20 kV. The spectrometer is equipped with a pulsed nitrogen laser operated at 337 nm with 3 ns duration pulses. MS spectra were acquired as an average of 100 laser shots. Peptide mass mapping was carried out using Findpept tools in ExPASy (Expert Protein Analysis system, <http://www.uniprot.org>).

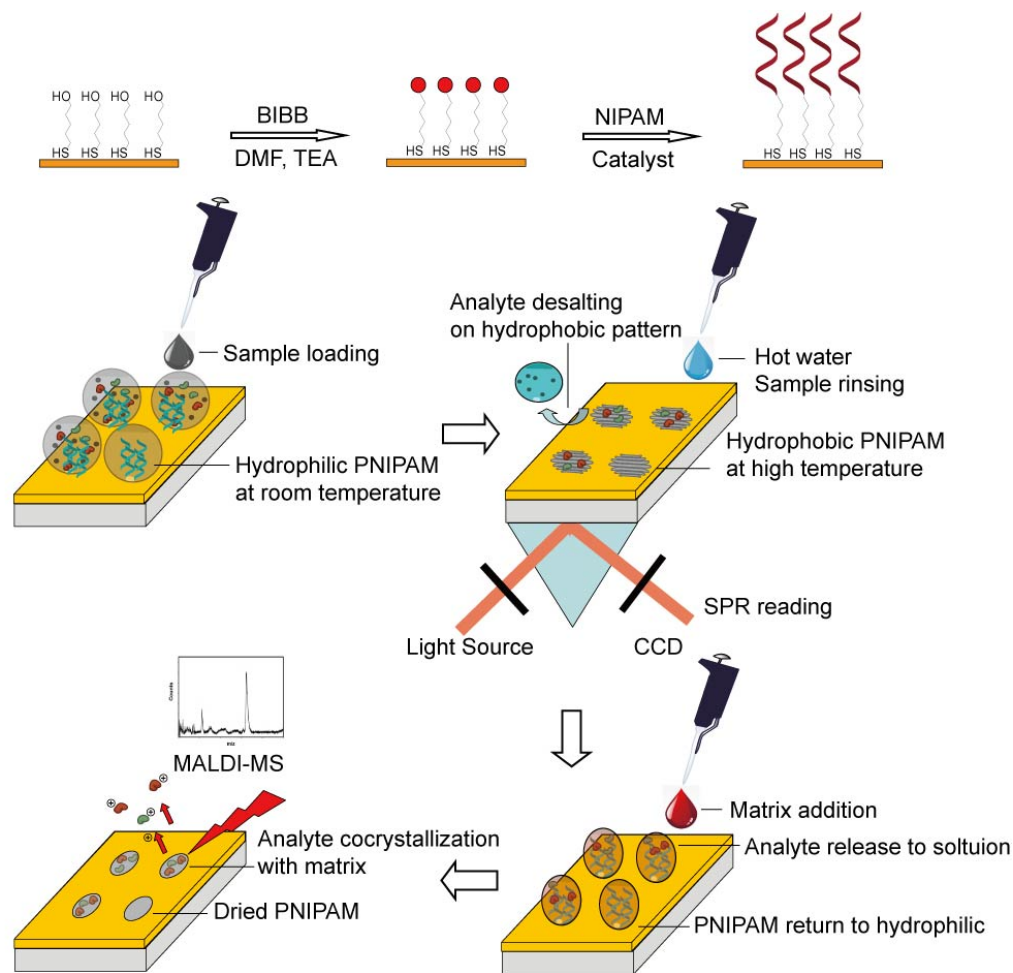


Figure 8.1. Cartoon representation of the PNIPAM growth from gold substrate via SI-ATRP reaction and on-plate desalting process based on PNIPAM polymer chain hydrophilicity change.

8.3 Results and discussion

8.3.1 Surface-initiated ATRP of PNIPAM on a gold surface at different temperatures

Surface-initiated atom transfer radical polymerization (SI-ATRP) procedures require the anchoring of small molecule initiators to surface, including thiol-gold bonding or silane-silane bonding²⁸. However, complicated synthesis steps were involved²⁹. An alternative route to generate silane initiator is to convert silanol (Si-OH) groups to primary amine groups by treating with 3-aminopropyltriethoxysilane (APTES); following reaction with BIBB converted the amine groups to initiators²⁶. In this work the initiators were generated from self-assembly monolayer of MCH. By reacting with BIBB, the surface immobilized hydroxyl groups were converted to initiator for ATRP reaction.

Atom force microscopy (AFM) and ellipsometry have been used to characterize the stimuli-responsive polymer grafted surface²⁴. Here we used SPR to monitor PNIPAAm growth at room temperature and 60°C respectively (Figure 8.2). The continual SPR signal increase during incubation showed the formation of polymer chain from surface. PNIPAAm growth at room temperature was demonstrated in Figure 8.2a. The hydrophilic polymer took the coil configuration and extended well into aqueous solution. The loose structure made water easily penetrated into the polymer chain space and resulted in 0.13 degree of SPR signal increase. In comparison, SPR signal showed a steep decrease during temperature increase to 60 °C due to water refractive index change³⁰. The

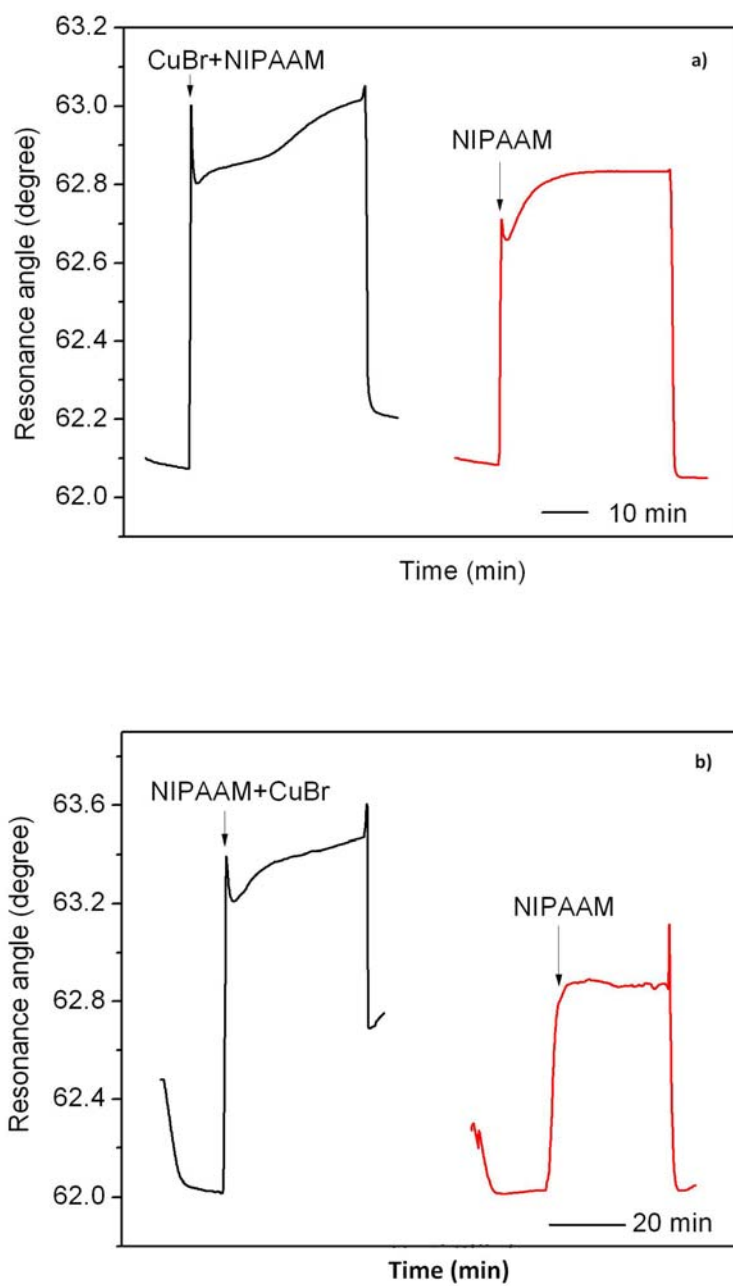


Figure 8.2. SPR sensorgrams of SI-ATRP PNIPAAm polymer growth from gold substrate a) at room temperature and b) at 60°C.

PNIPAAM polymer chain turned to hydrophobic and collapsed on surface at 60°C, its compact configuration enhanced polymer surface packing density efficiently, which resulted in 0.69 degree of SPR signal increase (Figure 8.2b). Control experiment was conducted in the absence of catalyst. There was no measurable SPR signal change at both temperatures, demonstrated good specificity of polymer growth and prevention of nonspecific adsorption from the monomer.

SPR was also used to *in-situ* characterize the change of grafted PNIPAAM polymer chain configuration. As shown in Figure 8.3, ATRP reaction was triggered on initiator immobilized surface at 60 °C, followed by PBS buffer rinsing to remove nonspecific adsorption of monomer or catalyst. The temperature was then decreased to room temperature by a temperature control device coupled to SPR spectroscopy. SPR signal was observed to increase at first due to water refractive index increase, followed by a sustained drop, because the hydrophobic PNIPAAM polymer chain turned to hydrophilic. SPR signal kept decreasing during this process until PNIPAM polymer chain well extended into solution. In the control experiment, there was no polymer growth in the absence of catalyst, thus SPR signal kept increase during temperature increase and returned to the initial level at room temperature.

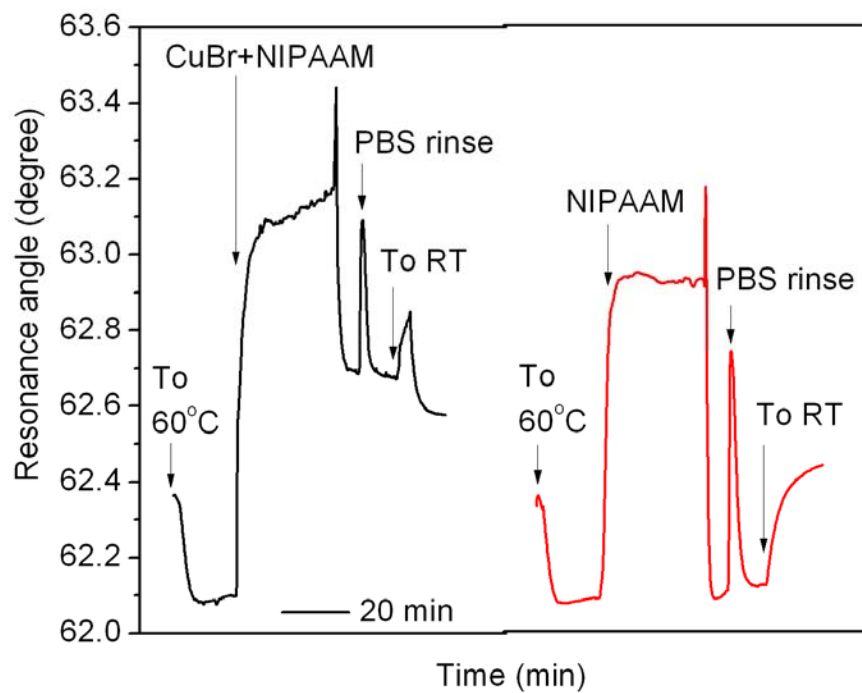


Figure 8.3. SPR sensorgram of PNIPAAm polymer chain configuration change according to temperature.

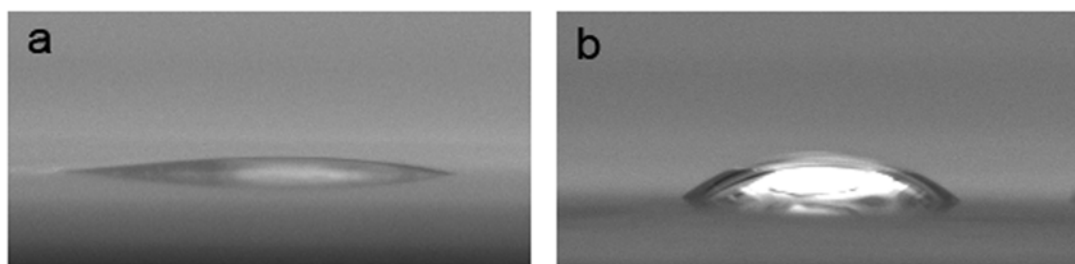


Figure 8.4. Contact angle measurement of PNIPAAm grafted surface a) at room temperature and b) 60°C.

8.3.2 Surface characterization

Surface hydrophobic property plays an important role in sample enrichment and desalting^{15, 16, 22, 31}. Contact angle measurements were carried out to evaluate the surface hydrophilicity at different temperature. The water droplet spread fast on hydrophilic PNIPAAm at room temperature, showed a contact angle of 6.1° (Figure 8.4a). PNIPAAm grafted substrate was then put on hot plate with temperature adjusted to 60°C, which resulted in a higher contact angle of 44.2°, indicating an increase in surface hydrophobicity (Figure 8.4b). The hydrophobic property of the surface provided control over protein/peptide adsorption/release, which may also assist the formation of homogeneous crystals on top of the polymer layer for MALDI-MS analysis.

8.3.3 Surface binding and desalting of protein and peptide

Cytochrome c was chosen as model protein to demonstrate protein adsorption/desorption on the polymer surface. The hydrophobic PNIPAM polymer chain has good performance to retain cytochrome c on surface, resulted in 0.28 degree of signal increase (Figure 8.5a). In comparison, cytochrome c only generated 0.03 degree of SPR signal increase on hydrophilic PNIPAAm surface at room temperature (Figure 8.5b). PNIPAM also demonstrated similar adsorption preference for neurotensin peptide (Figure 8.6). 0.2 mg/mL neurotensin was incubated on PNIPAM grafted surface for 1 hour to ensure sufficient binding, SPR signal slowly increased during incubation, demonstrated

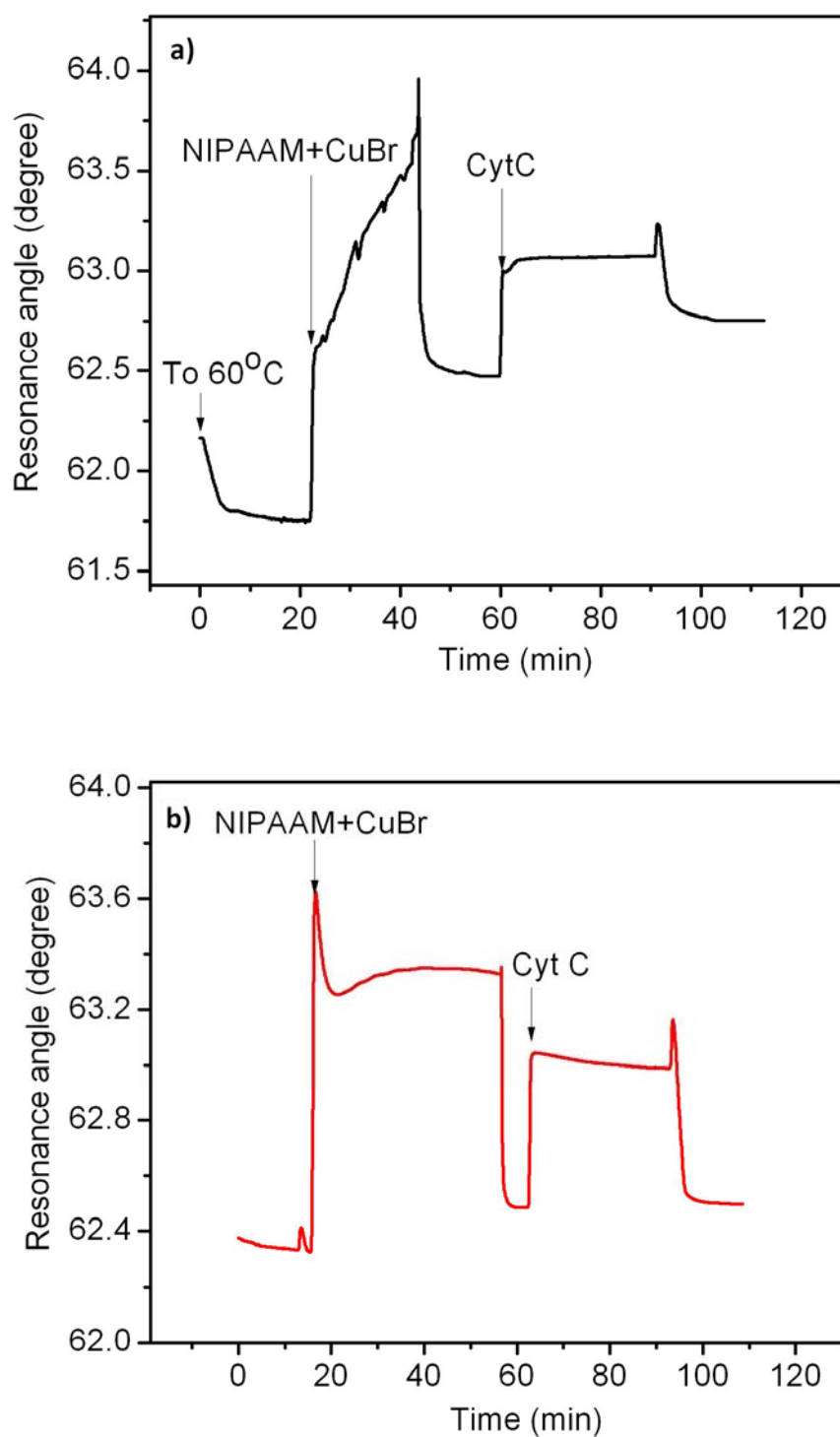


Figure 8.5. SPR sensorgrams for the adsorption of Cytochrome c on PNIPAAm at a) 60°C and b) room temperature.

the adsorption of peptide to polymer chain from solution. A 0.06 degree of SPR signal increase was observed on hydrophobic PNIPAM surface (Figure 8.6a). The increase is limited compared with cytochrome c adsorption due to the smaller molecular mass of neurotensin. SPR signal was stable during neurotensin incubation on hydrophilic PNIPAAm surface at room temperature, and demonstrated little signal increase after surface rinsing (Figure 8.6b).

An amphiprotic block copolymer polysulfone-poly(ethylene oxide) has been reported on-plate desalting²². The hydrophilic domain of poly(ethylene oxide) can strongly absorb salts, while peptides is concentrated and enriched with its hydrophobic domain of polysulfone. However, the enriched peptides need to be transferred to MALDI plate for MS analysis, which may cause sample lose or contamination. Taking advantage of PNIPAM hydrophilicity change at varied temperature, we performed on-plate desalting with the PNIPAM grafted substrate. Sample loading was conducted at room temperature, the hydrophilic PNIPAM chain acted as anchor to hold sample droplet in place. Then the substrate was rinsed with hot water. The hydrophobic PNIPAM polymer chain should retain protein/peptide on surface, while the salts and contaminants could be washed away. The matrix was then applied at room temperature; the hydrophilic PNIPAM chain can release peptide and co-crystallize with the matrix. MALDI-MS analysis was directly performed on matrix deposited spots. The on-plate desalting of PNIPAM surface was

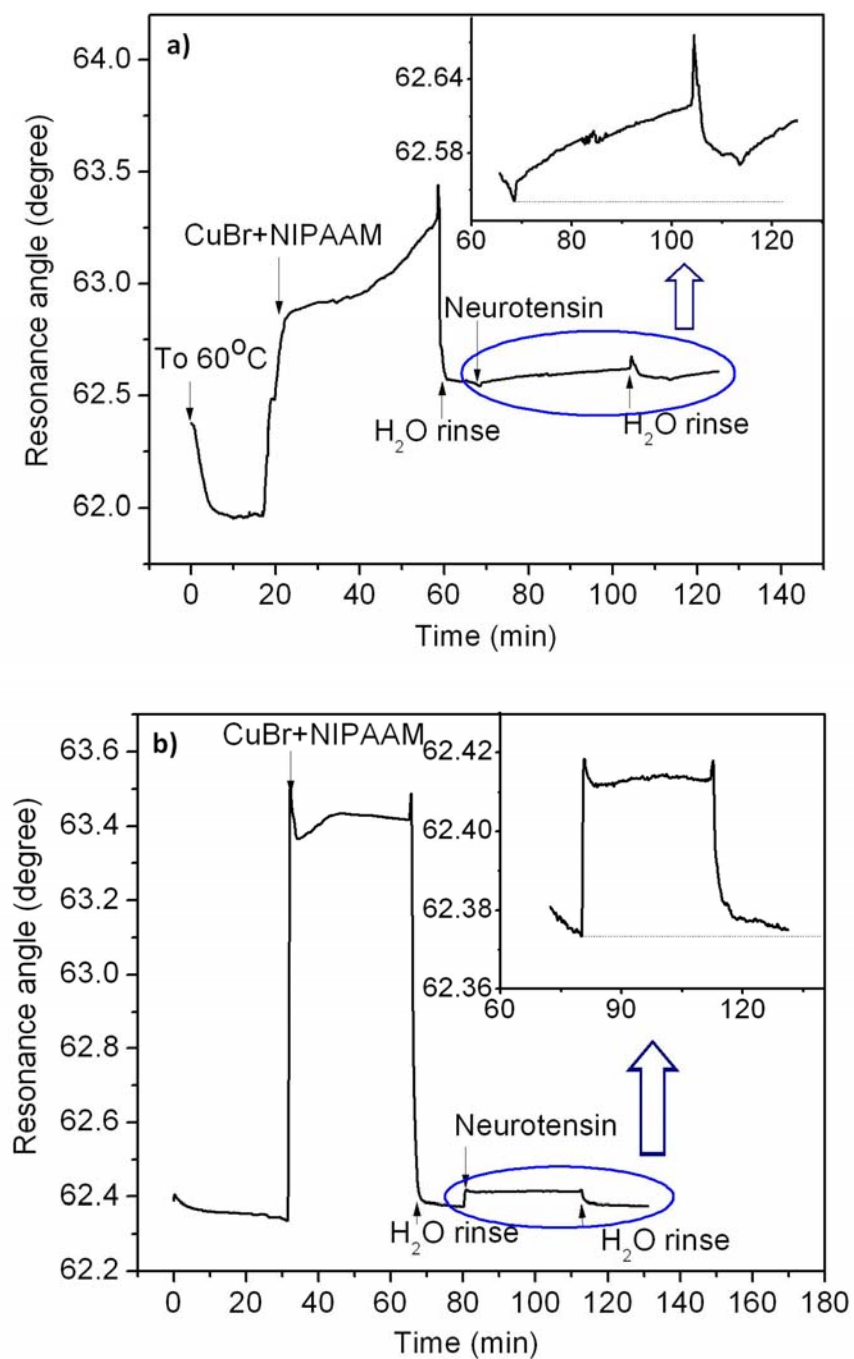


Figure 8.6. SPR sensorgrams for the adsorption of neurotensin on PNIPAM at a) 60°C and b) room temperature.

tested using 100 mM NaHCO₃. Figure 8.7 shows MS spectra for 120 pmol of neurotensin peptide with and without on plate desalting. For MALDI-MS analysis, high salt concentration highly affects the crystallization as many of these salts are incompatible with organic matrix solutions. Without desalting, a layer of salt precipitate was generated after sample drying on the sample spot; this layer is believed to largely impede the UV absorption and thus the ionization process. The high background noises were dominant in the spectrum, yielding poor detection sensitivity and no signal from the peptide (Figure 8.7a). After remove of the salt by hot water with on-plate washing, the neurotensin peptide peak was clearly identified with highly enhanced signal to noise ratio (Figure 8.7b).

8.3.4 Application for the analysis of protein digests.

PNIPAM on-plate desalting and MALDI-MS was further applied to the analysis of protein digests, and its performance was compared with commercial C18-HyperSep desalting tip. The operation of C18 desalting tip is laborious due to multisteps of sample handling including tip precondition, sample binding, sample washing and sample release, giving close to hundred time of pipetting. The PNIPAM on-plate desalting is much simpler, only five time pipetting was counted before MALDI analysis¹³. The performance of two desalting methods were tested with 2 pmol of tryptic digest of α -casein in the presence of 50 mM NH₄HCO₃, and the identified peptide peaks with their sequence and

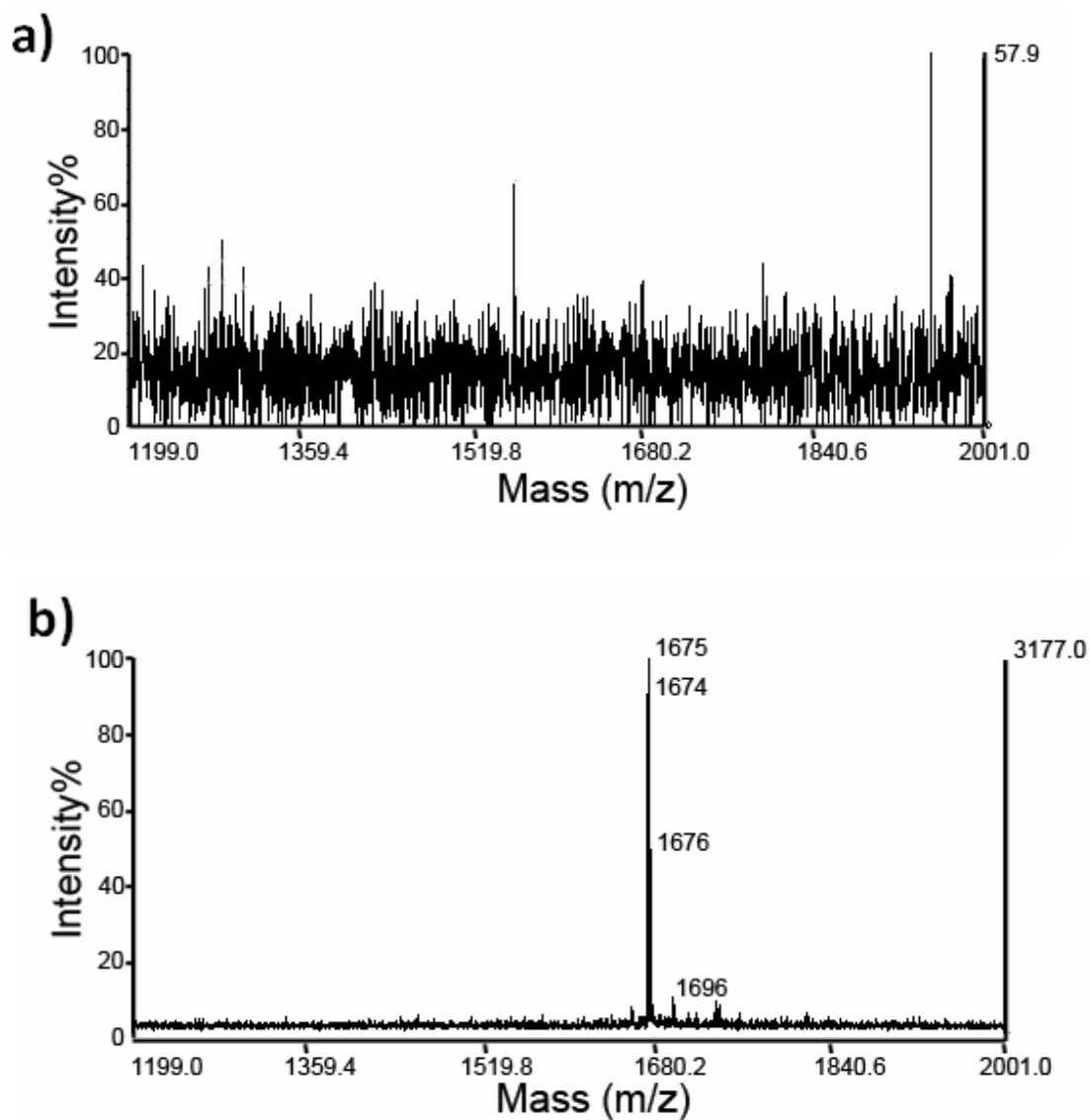


Figure 8.7. Mass spectra of 120 pmol neurotensin with 100 mM NaHCO_3 mixture solution a) before desalting on normal stainless MALDI plate and b) after PNIPAM on-plate desalting.

Table 8.1. Mass spectra of 2 pmol α -casein digests with C18 tip desalting and PNIPAM on-plate desalting.

MH ⁺	Amino acid sequence	Protein	Position	C18 desalting tip	PNIPAM on-plate desalting
973.1	YQKFPQY	α -S2-casein	104-110		*
991.4	ITVDDKHY	α -S2-casein	86-93	*	*
1133.5	EKNMAINPSK	α -S2-casein	198-206	*	*
1204.3	QKALNEINQF	α -S2-casein	94-103	*	*
1267.5	YLGYLEQLLR	α -S1-casein	106-115	*	*
1386.5	TVDMESTEVFTK	α -S2-casein	153-164	*	*
1407.7	REQLSTSEENSK	α -S2-casein	140-151	*	*
1759.9	HQGLPQEVLENLLR	α -S1-casein	23-37	*	*
1822.1	DQVKRNAVPITPTLNR	α -S2-casein	125-140		*
1855.7	HVS[pS]SEESII[pS]QETY	α -S2-casein	21-35		*

position were summarized in Table 8.1. On plate desalting with PNIPAM provides better result as compared to C18 tip method, with more peptide peaks identified. It is possible that some peptides were lost during the multiple pipetting process of C18 desalting tip³². This result clearly indicated the potential application of PNIPAM grafted substrate in high throughput proteome analysis.

8.4 Conclusion

We have reported the coupling of SPR and MALDI-MS for comprehensive biological analysis with PNIPAM grafted chip. The PNIPAM grafted surface is hydrophilic at room temperature, and hydrophobic at high temperature. It demonstrates different protein or peptide adsorption ability based on the surface property change. SPR was used to characterize PNIPAM polymer chain configuration change at different temperature, and quantitatively measure the amount of binding protein cytochrome c and peptide neurotensin. MALDI-MS analysis was directly performed on protein/peptide retained PNIPAM surface after on-plate desalting and addition of matrix at room temperature. The polymer coating was tested with the solution of 600 μ M neurotensin in 100 mM NaHCO₃, which yielded clear background with well recognized neurotensin peak. Furthermore, the PNIPAM on-plate desalting method has also been applied to 2 pmol α -casein digest sample, and the results were comparable to commercial C18 desalting tip.

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Chapter 9 Summary and Future Outlook

SPR has become the technique of choice for molecular interaction study and lead compound discovery, in particular for biopharmaceutical industry. The strategies we have developed and employed in this thesis will make SPR and related techniques more powerful. The major contributions in signal enhancement, novel surface design, and new application in lipid membrane systems have highly enhanced the performance of SPR, rendering it competitive to fluorescence methods for molecular detection.

A major breakthrough for the SPR system will be the wide acceptance of imaging SPR into the biotech industry. The rapid detection and profiling of multiple protein biomarkers in blood and serum samples can play an important role in the diagnosis of diseases and the monitoring of subsequent therapeutic treatments. In this sense, SPR_i is a more promising technique. However, major technical issues still hinder the broad use of SPR_i, despite the potential in high throughput capacity. Chapter 4 describes the ultrasensitive protein detection on an Au-well microarray via SPR imaging. The protein binding and SPR imaging signal amplification was demonstrated in a flow cell. To further extend the simultaneous multi-protein detection with the microarray, the research in Chapter 4 can be continued by the incorporation of a microfluidic system. Microfluidic systems have received considerable attention as tools that can bring significant benefits to drug discovery and development process. For high throughput analysis/screening, microfluidic

system has to be designed to accommodate multiple analyte spots in an addressable fashion. Different analytes could be injected to surface via multichannels, thus signal enhancement strategy can be implemented directly for detection of multiple analytes with ultra-high sensitivity.

Given the success in the development of SPR signal amplification via AuNP and ATRP in mild conditions, the research in Chapter 3 can be applied for new detection systems with aptamer microarray. Thiol-aptamer microarrays are promising because they have more stable physicochemical properties and are less susceptible to nonspecific adsorption than antibodies. Thiol-poly(ethylene glycol) would be used for surface blocking. Different biomarkers can be detected simultaneously from a mixed solution. The enhancement process would be carried out in an aqueous environment, ensuring the internal loop structures of aptamers and avoiding the loose of biomarkers from aptamer unfolding. We believe the novel SPR amplification strategy in mild conditions coupled with high-throughput analysis from Au-well array would provide a universal method for sensitively monitoring of proteins and biomarkers from biological solutions. For example, amyloid beta-protein ($A\beta$), the biomarker for Alzheimer disease (AD), is a self-assemble amphiphilic peptide that is nontoxic in its monomer form but becomes cytotoxic upon aggregation on cell membrane¹. Direct on-line SPR measurement with signal amplification can detect $A\beta$ in its native state and provide highly valuable information of

the interaction mechanisms between A β of various states and bilayer lipid membrane without interfering with its binding property. This could open doors to the studies of other misfolded proteins in disease, including Parkinson's, Huntington's, and type II diabetes².

To further explore the interaction on the cell lipid membrane, there is an increasing demand for cell membrane functionalization and control of the surface property. In Chapter 7, we first demonstrated polymer growth at the lipid membrane-water interface by membrane-bound cavitand and guest-initiator via a surface initiated ATRP reaction. In future, novel polymers could be used to functionalize cell surface for cell adhesion and proliferation. Surface wettability and roughness are two important parameters influencing cell behavior. Suitable polymer materials include peptide-incorporated polymers and glycopolymers. Polymer-peptide hybrid materials have been used to control cell adhesion, and the cells adhesion increase with wt% of peptide increase^{3, 4}. Glycopolymer 2-lactobionamidoethyl methacrylate (LAMA) has been grafted on surface via UV-induced graft polymerization⁵ and surface initiator ATRP reaction⁶ to selectively bind cells.

The coupling of analytical instruments with SPR biosensors has been explored with limited success in bioanalysis. In Chapter 8, we demonstrated the coupling of on-chip SPR with MALDI-MS for protein analysis. It involves a polymer grafted surface, and SPR is used to characterize the activity of a target molecule, on-chip desalting, followed

by direct MS analysis. This procedure enables rapid study of both the function (by SPR) and the structure (by MS) of the binding molecules. The research in this direction could be extended by incorporating other smart polymers for separation of peptides. Our group has demonstrated an ultrathin calcinated film on a gold SPR substrate allowing for effective laser desorption/ionization MS analysis of proteins and peptides without the need of an organic matrix⁷. The surface can be combined with polymer coating and interfaced with SPR to quantitatively monitor binding event. A highly interesting development is the creation of an SPR-MS array platform and realization of SPR/MS detection on a single high-content protein microarray⁸. Both protein concentration as well as structural aspects of protein variants can be detected. SPR-MS has been reported for automated affinity purification of recombinant and native proteins and identification of proteins captured on DNA surfaces⁹. We believe SPR-MS technical platform will continue to evolve and will provide more opportunities to meet the increasing demand in the development of the “-omics” technologies for high-throughput analysis of biomolecular interaction networks and structural identification.

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