Synthesis and Functionalization of Gold Nanoparticles Using Chemically Modified ssDNA

by

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A thesis submitted to the Faculty of the Graduate School of the University of Colorado in partial fulfillment of the requirements for the degree of Doctor of Philosophy Department of Biochemistry 2014 This thesis entitled: Synthesis and Functionalization of Gold Nanoparticles Using Chemically Modified ssDNA written by P. G. Calabrese has been approved for the Department of Biochemistry

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The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline. Calabrese, P. G. (Ph.D., Biochem)

Synthesis and Functionalization of Gold Nanoparticles Using Chemically Modified ssDNA Thesis directed by Prof. Bruce E. Eaton

In the first part of this thesis, methods for functionalizing spherical gold nanoparticles with nucleic acid binding ligands (aptamers) that target the VEGF receptor complex were developed. In order to provide a multiplexed labeling strategy for imaging the VEGF receptor complex in electron microscopy, gold nanoparticles of distinct sizes were conjugated to modified ssDNA aptamers that target the VEGF-A cytokine, the VEGFR-2 RTK receptor and a membrane associated co-receptor, Nrp-1. The modified ssDNA gold nanoparticle conjugates were applied to a human lung carcinoma cell line (A549) which has been shown to express each of these proteins and used as a model system for VEGF signaling. Binding constants for the modified aptamers were also determined using a fluorescence polarization anisotropy assay to determine K_D and K_{OFF} for the aptamers with their respective proteins.

In the latter part of this thesis, a modified ssDNA SELEX protocol was also developed in order to evolve imidazole modified ssDNA sequences that assemble gold nanoparticles from Au^{3+} precursor ions in aqueous solution. Active sequences bound to nanoparticles were partitioned from inactive sequences based on density via ultracentrifugation through a discontinuous sucrose gradient. Colloidal gold solutions produced by the evolved pool had a distinct absorbance spectra and produced nanoparticles with a narrower distribution of sizes compared to colloidal gold solutions produced by the starting randomized pool of imidazole modified ssDNA. Sequencing data from the evolved pool shows that conserved 5 and 6 nt motifs were shared amongst many of the isolates, which indicates that these motifs could serve as chelation sites for gold atoms or help stabilize colloidal gold solutions in a base specific manner.

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Contents

Chapter

1	Applications of DNA Functionalized Nanomaterials	1
2	Surface Modification of Gold Nanoparticles with Modified ssDNA Aptamers	8
3	Aptamer Nano-Gold Conjugates for Targeting the VEGF Receptor Complex	25
4	Imidazole Modified ssDNA Sequences that Template Gold Nanoparticles	49
5	Materials and Methods	69
F	Ribliography	81
1	Dibilography	

Figures

Figure

1.1	Applications of Bionanomaterials	2
1.2	Approaches for conjugating oligonucleotides to gold nanoparticles	5
2.1	Schematic of modified ssDNA SELEX process	10
2.2	Structures of dUTP modifications	12
2.3	Alkane thiols used in SAM.	14
2.4	Schematic Representation of SAM.	16
2.5	Migration of gold nanoparticles through 1.7% agarose gel	17
2.6	Emission spectra of fluorescent nanoparticle conjugates	18
2.7	Peaks from emission spectra plotted against percentage of 1, 8 ODT. \ldots	19
2.8	Schematic of bisphenylphosphine surface co-ordination.	20
2.9	Analysis of gold nanoparticles treated with or without DTPA aptamers	22
2.10	Analysis of DNA-nanogold conjugates purified by agarose gel electrophoresis.	22
2.11	8% Denaturing PAGE gel DTPA aptamers +/- gold	23
3.1	Differential binding modes of $VEGF_{165}$ and $VEGF_{121}$.	26
3.2	The tumbling rate of a fluorescent molecule in solution measured by FPA. $$.	30
3.3	Schematic representation of fluorometer setup	31
3.4	Structure of the CalFluor610 Phosphoramidite.	33
3.5	Dilution series of CF610 at 6 nm slit widths.	34

3.6	Peaks from the CalFluor610 serial dilution plotted versus concentration	35
3.7	Change in anisotropy of VEGF-A aptamer with addition of ligand. \ldots .	35
3.8	VEGF-A aptamer binding curve (top) and curve fit analysis (bottom)	38
3.9	Nrp-1 aptamer binding curve (top) and curve fit analysis (bottom)	39
3.10	FP as say sensitivity comparison of VEGF 30mer (left) and VEGF 50mer (right)	40
3.11	Anisotropy vs. Time in \mathbf{K}_{OFF} experiment (top) and curve fit analysis (bottom)	41
3.12	VEGFR-2 added after VEGF-A aptamer/protein complex established	42
3.13	Heparin sulfate added after VEGF-A aptamer/protein complex established .	43
3.14	TEM images of A549 cells treated with 5 nm gold - VEGFA aptamer and 10 $$	
	nm gold scrambled sequence	45
3.15	TEM images of A549 cells treated with 5 nm gold - VEGFA aptamer and 10 $$	
	nm gold- VEGFR2 aptamer	46
3.16	Number of nanoparticles per cell surface area	47
4.1	Modified ssDNA SELEX scheme for nanoparticle templating sequences	51
4.2	Structure of imidazole modified dUTP.	52
4.3	TEG-MUE protected gold nanoparticles sediment to the boundary of a 30%	
	over 67% sucrose gradient.	55
4.4	8% denaturing PAGE gel of 500 uL fractions from the top of the gradient. $% 10^{-1}$.	57
4.5	Common biological reducing agents facilitate the reduction of $\mathrm{Au^{3+}}$ to $\mathrm{Au^{0}}.$.	59
4.6	Round 0 imidazole modified DNA library(blue) vs. buffer(green)	61
4.7	Conditions of SELEX experiment in each round	62
4.8	PCR trace of +/- Au Samples (left), 8% denaturing PAGE gel of PCR reaction	
	(right)	63
4.9	Samples with and without ssDNA after 6 hours	64
4.10	Consensus sequences grouped into families amongst evolved round 8 pool	65

4.11	TEM images of round 8 imidazole modified ssDNA (A), round 0 imidizole	
	modified ssDNA library (B), and no ssDNA (C)	66
4.12	Size distribution of gold nanoparticles amongst rd.8 (red), rd. 0 (blue) and	
	no DNA (black).	66
4.13	Absorbance spectra comparing no DNA (red), 100 nM rd. 0 DNA (green),	
	100 nM rd.8 DNA (blue). A picture of the cuvettes used in the measurement	
	are shown on the right.	67

Chapter 1

Applications of DNA Functionalized Nanomaterials

Introduction

As our collective scientific understanding of biologically guided processes at the molecular level has developed alongside our ability to control the synthesis and functionalization of materials at the nanoscale, a crossroads where biology and nanotechnology intersect has emerged. Discoveries and developments in bionanomaterials have led to novel ways of delivering payloads in medicine, improved limits of detection and better specificity for diagnostics as well as biologically inspired methods towards constructing nanoelectronics. These advancements have been made possible by the functionalization of nanoscale surfaces with biorecognition elements as well as the engineering of biological guided processes to template or control the bottom up synthesis of inorganic materials from metal precursors [1]. Some of the various applications of bionanomaterials are shown in Figure 1.1.

In particular, gold nanoparticles have proven to be extremely useful in applications within bionanotechnology because of the unique electronic, magnetic and optical properties they exhibit, which are attributed to quantum size related properties that gold clusters on the order of 1 - 100 nm in size exhibit [2]. Colloidal gold is a fluid suspension of particles which are unlike individual atoms or bulk metal but rather behave as zero-dimensional quantum dots. Within the nanoparticle's physical boundaries, freely mobile valence electrons, whose *de Broglie* wavelength is on the order of the size of the particle itself, collectively oscillate



Figure 1.1: Applications of Bionanomaterials.

within the boundaries of the particle and give rise to a plasmon resonance band near 530 nm for gold particles between 5 - 20 nm, owning to the ruby red color often associated with colloidal gold solutions. One of the most famous historical examples of colloidal gold's mysterious properties is the Lycurgus cup from ancient Greece, which changes color from green to red in when put in the path of light [3].

Investigations of colloidal gold's unique properties have been under scientific observation as far back as 1856 when Micheal Faraday discovered that metallic colloids deposited on thin films would change color based on tensile or compressive forces experienced by the film [4]. This simple example illustrates how the properties of colloidal gold can be tunable in solution due to a strong dependence on the interparticle distance. Other factors such as the particle size, morphology, and the nature of protecting organic shell also dictate their optical, electronic and magnetic properties. There have been over 10,000 publications focused on gold nanoparticles in scientific peer reviewed journals within the past decade and growth in this field continues to expand rapidly.

When applied to biological applications, gold nanoparticles are often functionalized

with peptides, antibodies or nucleic acids in order to direct nanoparticles to a target of interest in an experiment. For instance, target labeling of specific macromolecules in electron microscopy relies on such strategies to provide a recognizable, electron dense tag at the protein of interest when it is under investigation. Typically, antibodies are used to achieve this but single stranded oligonucleotides (aptamers) offer certain advantages over antibodies since they are significantly smaller, bind to their targets with better specificity in some instances and can be readily synthesized with functional groups that make them amenable to gold nanoparticle surface conjugation. The first two chapters of this thesis will focus on functionalizing gold nanoparticles with modified ssDNA aptamers and their application towards cell labeling strategies in EM.

The functionalization of gold nanoparticles with nucleic acids has led to a myriad of applications including optically based assays to detect SNP mutations, carriers for antisense RNA molecules to deliver payloads in medicine and cell imaging strategies for both light and electron microscopy [5, 6]. Seminal research on the use of thiolated oligonucleotides to functionalize gold nanoparticles was undertaken by the Mirkin lab at Northwestern University and the Alivisatos lab at Berkely in the mid-to-late 1990's. By using thiolated oligonucleotides to form a protective, self assembled monolayer around the nanoparticle surface, Mirkin, et al were able to demonstrate that they could program hierarchical assemblies of nanoparticles using DNA base pairing, pioneering the concept of directed assembly of nanoparticle arrays using biological programming [7]. This concept was further developed into an assay for detecting SNP mutations in a DNA sample based on optical changnes in solutiuon that occur due to nanoparticle aggregation mediated by temperature sensitive Watson-Crick interactions that define the interparticle distance in solution [6].

Approaches for decorating gold nanoparticles with nucleic acids to modulate cell behavior are often designed around the delivery of siRNA functionalized gold nanoparticles to cells in order to knockdown expression of a certain mRNA transcript. The siRNA is typically conjugated to the nanoparticle surface via a gold thiol bond or adhered to the surface though electrostatic interactions. Polyethylene glycol or other passivating molecules that help stabilize gold nanoparticles from aggregating in high ionic strength buffers or cell culture media are also included. A few of the major advantages to this approach are that siRNA functionalized gold nanoparticles offer a non-cytotoxic carrier for siRNA which imparts a greater half life compared to naked dsDNA and does not require the use of a transfection agent to cross the cell barrier [8].

Aside from the use of DNA base pairing and siRNA to functionalize gold nanoparticles for biological applications, aptamers can be conjugated to gold nanparticles in order to direct them to a protein target of interest as either a method of detection or a strategy for inducing receptor mediated nanoparticle uptake. For example, through specific binding of aptamers directed toward platelet-derived growth factor, cells that over-express PDGF accumulated PDGF aptamer-gold nanoparticle conjugates in their cytoplasm. Upon photoillumination, cells which contained gold nanoparticles allowed the differentiation of cancer cells from normal cells as they exhibited intense light scattering under a dark field optical microscope [9].

A variety of conjugation chemistries have been developed which enable nucleic acids to attach to gold surfaces. This is achieved either directly through an Au-S bond by using 5' terminal thiolated oligonucleotides or by placing a chemical group within a monolayer on a gold surface that selectively reacts with a labile derivative incorporated within or at the terminal end of a nucleic acid. Figure 1.2 shows a general scheme for using thiolated oligonucleotides (top) and selective reactive groups (bottom) to attach tether DNA to gold nanoparticle surfaces.

Two strategies for functionalizing gold nanoparticles with aptamers are presented in Chapter 1. We show the successful conjugation of a dithiol phosphoramidite terminated aptamer to gold nanoparticles as well as a mixed monolyer gold nanoparticle surface passivation strategy that can covalently link malameide derivatized molecules to the nanoparticle surface through the formation of a thioether bond. The aptamer nano-gold conjugates were



Figure 1.2: Approaches for conjugating oligonucleotides to gold nanoparticles.

applied to cells with surface receptors that were targeted by the aptamers and investigated with electron microscopy. The results of this experiment along with studies of the binding interactions between modified ssDNA aptamers and their respective target proteins with fluorescence polarization anisotropy will be discussed in Chapter 2

Nucleic Acids as Biotemplates for Nanoparticle Assembly

In the third chapter of this thesis, a materials based in-vitro SELEX experiment is presented where the objective was to evolve imidazole modified ssDNA sequences that assemble Au^{3+} ions into gold nanoparticles. The parameters of the SELEX experiment were adjusted such that sub micromolar concentrations of Au^{3+} could be assembled into stable colloidal gold nanoparticles in an aqueous, buffered solution with the help of imidazole modified ss-DNA and a mild reducing agent. We envisioned that ultimately the evolved isolates could be used as fusion sequences to protein binding aptamers and nucleate gold nanoparticles under mild reaction conditions at the site of an aptamer bound to a protein.

We chose imidazole as a base modification due to previous reports that implicate histidine as a crucial residue in the templating of gold nanoparticles with certain peptide sequences that were used in bionanomaterials synthesis applications [10]. For example, the repeating consensus sequence AHHAHHAAD from the histidine-rich protein II of *Plasmod-ium falciparum* was reported to mediate the aqueous self-assembly of zerovalent gold metal clusters with the aid of a reducing agent. This same sequence was subesquently shown to mediate the assembly of gold nanowires after being immobilized on heptane dicarboxylate nanowires. The imidazole side chain offers an electron donating nitrogen that has been shown to form coordination complexes with Au^{3+} and other metal cations [11].

With the impressive amount of progress that has been made regarding gold nanoparticle synthesis and functionalization, no examples to our knowledge exist which utilize *in-vitro* selection of ssDNA to discover sequence specific biotemplates for gold nanoparticle assembly. There have, however, been examples of nucleotide and nucleic acid templates for the synthesis of a variety nanoparticles such as tRNA templated CdS quantum dots [12], nucleotide capped gold nanoparticles [13], and Ag nanocluster formation templated by cytosine oligonucleotides [14]. However, all of these approaches require high concentrations of metal precursor and template nanoparticles in non-sequence specific ways.

One route towards the discovery of sequence specific oligonucleotide templates that can assemble metal or metal oxide nanoparticles is the use of a materials based SELEX approach. In this approach, sequences which remain bound to nanoparticles after an incubation step are partitioned from unbound sequences, which are considered to be inactive, and amplified. In the first SELEX based experiment of its kind, RNA sequences capable of mediating the formation of palladium and platinum nanoparticles from metal precursor ions found through *in-vitro* SELEX were found to assemble nanoparticles with control over shape and crystallinity [50]. An advantage of using SELEX for nanoparticle synthesis is that evolved sequences can work with low concentrations of metal precursor, which at higher

Biomolecules that can cluster electron dense nanoparticles from precursor ions offer new ways to address limitations in the bottom up synthesis of nanoscale materials as well as improvements upon methods used in the investigation of biological systems. For example, the ferritin protein, whose function it is to store iron within a core of 24 protein subunits, has been used as an electron dense tag to follow proteins in electron microscopy. [15]. Biomolecules that can template nanoscale crystallization processes at physiological pH and temperature have also been used as parts in bottom-up assembly strategies for hybrid nanoscale materials that can be programmed to position biological components into hierarchical assemblies. For example, the Belcher lab at MIT has created new routes for the assembly and synthesis of nanowires for lithium ion battery electrodes using genetically engineered viruses with high affinity sites for certain components of the battery that template nanoparticles. By incorporating gold-binding peptides into the filament coat, they were able to form hybrid goldcobalt oxide wires that improved battery capacity [16].

concentrations might otherwise precipitate with other components of a biological sample.

We set out to discover ssDNA sequence motifs that nucleate gold nanoparticles using low concentrations of gold precursor in aqueous, buffered reaction conditins. Ultracentrifugation was used during the selection step in order to select for sequences bound to gold nanoparticles and discard unbound sequences based on their difference in sedimentation velocity through a discontinuous sucrose gradient. Evolved sequences produced nanoparticles of smaller size with tighter size distributions compared to a random library or without the use of DNA.

Chapter 2

Surface Modification of Gold Nanoparticles with Modified ssDNA Aptamers

Introduction

History, Development and Applications of Aptamers

The scope of this chapter will focus on functionalizing gold nanoparticles with ssDNA aptamers, which are nucleic acid binding ligands discovered through an *in-vitro* evolutionary process. *In-vitro* evolutionary processes select and amplify biomolecules with a desired trait amongst chemically, structurally and functionally diverse populations of oligonucleotide or peptide sequences. These methods are often used to discover ligands that bind with high affinity and specificity to desired biological targets. The modified ssDNA aptamers used in the conjugation stratagies outlined in this chapter contain aromatic hydrophobic modifications at each uracil base, which incorporate functional groups such as benzyl, napthyl and indole at the 5 position of each 2' deoxy uridine incorporated in the oligonucleotide.

The Joyce lab at Scripps Research Institute was the first to publish a technique whereby a researcher could mutate, select and amplify RNA molecules with desired catalytic properties as they reported the discovery of sequences that were capable of catalyzing a *trans* splicing reaction between themselves and an RNA substrate. By way of its intrinsic genotypic and phenotypic properties, they determined RNA could be a substrate for molecular evolution in the laboratory [17]. Later that year, two labs independently developed a technique of RNA *in-vitro* selection whereby sequences from a library of 10^{14} randomized oligonucleotides were evolved through iterative cycles of an applied selective pressure followed by reverse transcription and PCR amplification. The Gold lab at the University of Colorado published a paper in Science describing a process of selecting RNA ligands that bind T4 DNA polymerase [18] while coining the acronym SELEX (Systematic Evolution of Ligands through EXponential enrichment). Meanwhile, the Szostak lab at Harvard published a paper in Nature where they described a similar *in-vitro* process for selecting RNA ligands against organic dyes [19]. The Szostak lab coined the term aptamer, a clever portmanteau of two greek words, which respectively mean to fit (aptus) and part (meros). Aptamers have held certain advantages over antibodies because they can be chemically synthesized easily, they do not require the use of animals, they can denature and renature into their native structures unlike most proteins and they do not stimulate the auto-immune respone, which is one of the major side effects of antibody based therapies.

SELEX based approaches present a large population of unique nucleic acid sequences that create diverse structural libraries of oligonucleotides which are generated by randomizing sequence space over a stretch of typically 40 to 100 nt. Since the primary sequence dictates secondary and tertiary interactions within a molecule, access to a diverse library of approximately 10¹⁴ unique sequences is achieved by synthesizing one nanomole of a randomized library, which can be practically achieved through standard phosphoramidite synthesis on a DNA synthesizer. The ssDNA library is then subjected to two cycles of PCR to generate a dsDNA library that can be synthesized into the starting pool of RNA or ssDNA enzymatically. In traditional RNA based selections, the RNA library is generated using T7 RNA polymerase and an appropriate primer that contains the T7 start site. In modified ssDNA based selections, which is the only variety of aptamers and SELEX this thesis will cover, the dsDNA library is immobilized on streptavidin-agarose resin by using a biotinylated PCR primer during the generation of the starting pool and at each round of selection. The native sense strand is eluted and modified ssDNA is generated by a thermostable polymerase that incorporates 5' modified UTP derivatives at each uracil base during a primer extension step (Figure 2.1) [20].



Figure 2.1: Schematic of modified ssDNA SELEX process.

There are numerous variations to the aforementioned examples of RNA and DNA based SELEX protocols which would be incredibly exhaustive to list and outside the scope of the work presented here. Aptamer discovery and development has undergone sustained progress both in academic and biotechnology labs across the world for the past twenty years. Of note were advancements that made aptamers more feasible in clinical practice by utilizing phosphate backbone modifications which confer resistance against nucleases such as 2' fluoro, 2' O-methyl and phosphorothioate modifications [21]. The phosphorothiote bond, for example, substitutes a sulfur atom for a non-bridging oxygen in the phosphate backbone of an oligonucleotide, rendering the backbone resistant to nuclease degradation. Nuclease resistance as well as other modifications to the DNA backbone and its constituent nucleotides have helped overcome a major hurdles for making aptamers viable in human patients and amenable to new chemical functions. While there is currently only one FDA approved aptamer based treatment, Macugen for macular degeneration, [22] there are numerous academic and industrial biotechnology labs seeking to further develop their applications in human patients.

Aside from backbone modifications, the incorporation of modified dNTP and NTP derivatives into SELEX protocols enhances the chemical diversity and sequence space available to the nucleic acid library by introducing new functional groups that bestow properties such as hydrophobicity, acid-base catalysis and metal chelation [20]. The modified ssDNA aptamers used in the conjugation stratagies presented in this chapter contain benzyl, napthyl or indole modifications, respectively, at each uracil. Structures of these dUTP modifications are shown in Figure 2.2. Such modifications introduce functional groups that are absent in A, T, G and C but are often found in protein-protein, small molecule-protein, and antibody-antigen interactions. Modified aptamers as they appear to have distinguishable properties from traditional aptamers. This was exemplified by the fact that proteins which were previously unable to be selected against using native ssDNA were able to be selected against using ssDNA equipped with hydrophobic modifications [23]. A molecular level picture of a potential basis for this phenomena lies in the structure of PDGF co-crystallized with its modified aptamer ligand which reveals a hydrophobic binding surface that is not traditionally seen in protein binding aptamers which have mostly polar interactions [24].

Gold Nanoparticles Towards Biological Applications

When applied to biological applications, gold nanoparticles most often provide a stable core for the coordination of self assembled monolayers (SAMs) of organic molecules which enables an investigator to tune the hydrophobicity, charge, reactivity and bioconjugation strategy towards a desired application [25]. In order to render gold nanoparticles stable in these environments, a protective barier can be provided through the adsoption of a highly ordered, tightly packed, self assembled monolayer of thiolated ligands on the gold surface. Alkane thiols with repeating polyethyle glycol units have been shown to assemble into stabilizing monolayers which confer solubility in biologically buffered systems and provide a



Figure 2.2: Structures of dUTP modifications.

neutral, non-specific surface [26]. Alkane thiols are also highly compatible to ligand exchange reactions with citrate stabilized colloidal gold nanoparticles, which can be obtained inexpensively and then tailored towards a specific application.

Biofunctionalized gold nanoparticles have provided new inspiration for solving some of the most complex challenges in our ability to treat and diagnose disease. Gold nanoparticles can be used as non-toxic carriers for drug and gene delivery applications offering better payload delivery and longer half-lives to the bioactive component conjugated or adsorbed to the surface. We set out to conjugate modified aptamers that have enhanced hydrophobic binding properties with biocompatible gold nanoparticles in order to target the VEGF receptor protein complex with our aptamer-nanogold conjugates. Strategies whereby modified ssDNA aptamers could be conjugated directly to the gold surface or to a stabilizing ligand bound to the nanoparticle surface containing a reactive sulfhydryl were developed. Since the aptamers were selected against purified recombinant protein targets, it was unknown what the binding interface between the aptamer and protein was or if hydrophobic modified aptamers were able to recognize their targets within the context of the cell. We also did not know how or if the modified aptamers would associate or otherwise interact with the gold nanoparticle or the ligands pre-bound to the surface.

Experimental and Results

Mixed Self Assembled Monolayer of 1, 8 Octane Dithiol and Triethyleneglyclol 11-Mercaptoundecanoic Ether

Self assembled monolayers are ordered molecular assemblies formed spontaneously on surfaces by adsorption. Alkane thiols are commonly used in creating self assembled monolayers on gold surfaces because they can pack tightly amongst one another while coordinating to the gold surface through an Au-S bond. The first strategy for nanoparticle surface passivation and aptamer conjugation presented utilizes a self assembled mixed monolayer of 1, 8 octane dithiol and triethylene glycol mercaptoundecanoic ether. Triethyle glycol mercaptoundecanioc ether, from now on referred to as PEG₃ alkane thiol or TEG-MUE for simplicity's sake, has previously been shown to pack tightly into self assembled monolayers on both flat and spherical gold surfaces. A PEG₃ alkane thiol, such as the one we utilize in this experiment, confers water solubility through 3 repeating ethylene glycol units and serves as a non-specific neutral passivating ligand on nanoparticles used for biologically based applications [26]. Structures of the alkane thiols used in the self assembled monolayer (SAM) are shown in Figure 2.3.

1, 8 octane dithiol (1, 8 ODT) can be treated as the reactive commponent in the mixed monolayer. We chose this ligand because 1, 8 octane dithiol should pack tightly within PEG_3 alkane thiols into a self assembled monolayer and present a reactive moeity within a core of a more fluid PEGylated layer of unreactive molecules. A thiol specific derivative, such as a malaiemide, readily forms a thioether covalent linkage with with thiols between pH 6.5 and



Figure 2.3: Alkane thiols used in SAM.

7.5.

Determining the relative percentage of each passivating ligand when forming the mixed monolayer around the gold nanoparticle surface was the first step taken in developing our aptamer-nanoparticle conjugation strategy. Our objective was to create a stabilizing monolayer that would render the nanoparticles resistant to aggregation in high ionic strength solutions while also maintaining a reactive component within the monolayer that was amenable to further conjugation to a maleimide derivatized molecule. A detailed description of the mixed monolayer protocol can be found in Chapter 5. Briefly, 1 mM solutions of each thiolated ligand were prepared in 100% ethanol, mixed at different ratios and added to an equal volume of 10 nm citrate stabilized gold nanoparticles in Milli-Q H_2O . Citrate is a weak passivating agent that keeps colloidal gold solutions. The ligand exchange reactions were allowed to incubate overnight at room temperature with gentle agitation. The next day, nanoparticles were subjeted to repeated centrifugation and washing steps. Excess unreacted thiolated ligand in ethanol found in the supernatant was removed during this process and the particles were buffer exchanged into Milli-Q H_2O . A series of ligand exchange reactions were prepared such that the concentrations of 1, 8 ODT was increasingly titrated in order to determine the percentage of 1, 8 ODT needed in a PEG₃ alkane thiol monolayer for nanoparticle solutions that were monodisperse and able to present reactive thiols at the surface. At first, a wide range (0%, 10%, 20%, 40% and 60%) of relative 1, 8 ODT percentages were attempted. However, when the percentage of 1, 8 ODT was greater 10% in the ligand exchange reaction, the nanoparticle solutions quickly turned from red to purple to a black precipitate. This indicated that when 1,8 ODT was greater than 10% in solution during the ligand exchange reaction, it promoted aggregation by bonding multiple gold nanoparticles at once, thereby lowering the interparticle distance and turning the solution clear. The same observation was also made whenever 1, 8 ODT was added to citrate stabilized nanoparticles before the PEG₃ alkane thiol, suggesting that the presence of 1, 8 ODT by itself will quickly and irreversibly aggregate gold nanoparticles.

After these observations were made, we determined that working percentages of 1, 8 ODT between 0% and 10% would be suitable for our application. A series of ligand exchange reactions were prepared in order to determine whether control over the number of reactive thiols present on the nanoparticle surface could be achieved. This approach would be advantageous because having the ability to place a limited number of reactive groups on the nanoparticle surface would enable us to ultimately have better control over the number of aptamers conjugated to the nanoparticles. A detailed experimental protocol of the ligand exchange reaction can be found in Chapter 5.1.1.

The resultant nanoparticles were characterized by agarose gel electrophoresis and a fluorescence based assay. Since colloidal gold absorbs strongly in the 500 - 600 nm range, the gold nanoparticles could be visually tracked in an agarose shift assay and imaged directly on an image scanner. The fluorescence based assay was designed to detect the presence of malameide linked fluorophores that had formed a covalent thioether linkage with a reactive thiol placed on the nanoparticle surface. Figure 2.4 shows a schematic 2D representation of the mixed monolayer strategy.



Figure 2.4: Schematic Representation of SAM.

Agarose Gel Electrophoresis of Mixed Monolayer Nanoparticle Preparations

A difference in particle mobility in an agarose gel is observed when varying the percentage (0% - 10%) of 1, 8 octane dithiol in a mixed self-assembled monolayer of PEG₃ alkane thiol (Figure 2.5). After running the gel in 0.5X TBE for 2 hours at 100V, the agarose gel was removed from buffer, rinsed in Milli-Q H20 for 60 seconds and imaged directly on an EPSON scanner. The lanes are labeled according to the percentage of 1,8 ODT added to the ligand exchange mixture. The mobility of the nanoparticles through a 1.7% agarose gel increases with increasing 1, 8 ODT concentrations. Since 1, 8 ODT increases the negative charge and lowers the mass of the SAM on the gold nanoparticle surface, we would expect this result since it is consistent with the principle of gaining faster mobility through an



agarose gel with a greater charge to mass ratio.

Figure 2.5: Migration of gold nanoparticles through 1.7% agarose gel.

Conjugation of Fluorescent Malaimide to Surface Bound Thiols

In order to test the utility of conjugating malaimide derivatized molecules to our SAM on the gold nanoparticle surface, we set up a fluorescence based assay using an AlexaFluor₄₈₈ C-5 maleimide (Invitrogen). We used this fluorophore since its excitation and emission spectra do not overlap significantly with the absorbance maximum of 10 nm gold particles and therefore we should not observe fluorescence quenching. Gold nanoparticle-fluorophore conjugates were excited at the fluorophore's excitation maximum and the emission spectra was captured using the lab fluorometer. A detailed description of the reaction conditions and incubation protocol can be found in Chapter 5.1.3. Briefly, 500 uL of an 8 uM aliquot of AlexaFluor₄₈₈ was prepared from a 4 mM stock solution in pH 7.0 Milli-Q H₂0. 100 uL of 8 uM AlexaFluor₄₈₈ was added to 100 uL of 7 nM solutions of gold nanoparticles from the aforementioned mixed monolayer assembly protocol in Milli-Q H₂0, pH 7.0. The reactions sat at room temperature for 90 minutes. Excess, unreacted fluorophore was washed away by repeated centrifugation and resuspension in Milli-Q H₂0 through 100K MWCO membrane spin filters. A 1:20 dilution of the final, buffer exchanged reaction mixture was pipetted into a 200 uL quartz cuvette and placed in the lab fluorometer.



Figure 2.6: Emission spectra of fluorescent nanoparticle conjugates.

The nanoparticle solutions for each of the SAMs tested were excited at their excitation maximum of 488 nm and their emission spectra from 508 nm to 640 nm was collected. Emission spectra of 0% (blue), 1% (red), 2.5% (green), 5% (purple) and 10% (black) 1, 8 ODT in PEG3 alkane thiol SAMs on 5 nm diameter gold nanoparticles. Figure 2.6 illustrates a clear relationship between the emission maximum intensity at 516 nm and increasing concentration of 1, 8 ODT within the SAM. Since maleimide groups react readily with thiols between pH 6.5 and 7.5 to form a covalent thioether linkage, we would expect this result if there were in fact more reactive 1, 8 ODT molecules present at the nanoparticle surface for the malaimide to react with. The background subtracted peak intensities at 516 nm for each nanoparticle preparation were plotted against the percentage of 1, 8 ODT in the SAM.

Figure 2.7 demonstrates that by controlling the relative amount of reactive groups



Figure 2.7: Peaks from emission spectra plotted against percentage of 1, 8 ODT.

on the nanoparticle surface, we can control the number of malaimide linked molecules that covalently bond to the surface. This strategy seemed worth developing further to covalently attach malamide derivatized aptamers to gold nanoparticle surfaces. We attempted to do this with a heterobifunctional crosslinker (Sulfo-GMBS) that reacts selectively on one end with primary amines and on the other end with thiols. However, we could not get 5' amine terminated aptamers to couple with the Sulfo-GMBS crosslinker before attempting to subsequently conjugate them to thiol derivatized nanoparticles. A potential reason for this was that in the first step of the protocol, the amine containing molecule must be kept at concentrations near 0.1 mM in order to react with the heterobifunctional crosslinker but some modified ssDNA aptamers, especially those containing hydrophobic modifications, are known to aggregate at concentrations greater than 5 uM.

Bis (p-sulfonatophenyl) Phenylphosphine and Dithiol Phosphoramidite Terminated Aptamers The other gold nanoparticle aptamer conjugation strategy utilizes a slightly different approach by first performing a ligand exchange on citrate stabilized nanoparticles using bis (p-sulfonatophenyl) phenylphosphine dihydrate dipotassium salt. This ligand provides a stabilizing, negatively charged monolayer around gold nanoparticles by coordinating to the Au surface through negatively charged sulfonate groups [27]. When arranged as a SAM on gold surfaces, it confers stability in aqueous solution under physiological conditions. Under mild reaction conditions, this ligand can be displaced by thiols or other reactive groups that have a stronger affinity for gold surfaces while the remainder of the SAM will remain intact. It is useful for thiolated aptamer conjugation stratagies since it prevents the nucleotides and phosphate groups on the DNA backbone from nonspecifically binding the gold surface, which would otherwise interfere with the binding properties of the aptamer.



Figure 2.8: Schematic of bisphenylphosphine surface co-ordination.

A disulfide-containing phosphoramidite (DTPA) was used to modify aptamers at the 5' end during the final coupling step of DNA synthesis. Each DTPA monomer forms two gold sulfur bonds, providing a more stable linkage to the nanoparticle surface than a monothiolate. The DTPA modification was included at the 5' terminus of the aptamers. After passivating 10 nm diameter gold nanoparticles with bis (p-sulfonatophenyl) phenylphosphine, a ligand exchange reaction was performed by incubating the nanoparticles with a 0.9X molar ratio of 5 terminated DTPA2 aptamers (Figure 2.8). The resulting nanoparticles were characterized by agarose gel electrophoresis and the conjugation of DTPA2 aptamers was confirmed by 8% denaturing PAGE, the results of which are shown in the next section.

Bis (p-sulfonatophenyl) Phenylphosphine, DTPA Aptamer Conjugation

Figure 2.9 shows the difference in migration through a 1.5% agarose gel between bis (psulfonatophenyl) phenylphospine protected nanoparticles treated with or without DTPA2 aptamers. Citrate stabilized nanoparticles not treated with bis (p-sulfonatophenyl) phenylphospine or DTPA aptamers did not migrate into the gel and formed a black precipitate in the well of the gel within 5 mintues. The band containing the aptamer-nanoparticle conjugates faintly contained two bands within it during the beginning of the run. In order to see if this band could be further purified into nanoparticle solutions containing discrete DNA-Au conjugates, the upper portion and lower portion were cut out from the agarose gel and subjected to passive elution to recover the nanoparticle-DNA conjugates. Figure 2.10 shows a 1.85% agarose gel comparing the difference in migration between nanoparticles untreated with DTPA-aptamers (1), treated with a 0.9X molar ratio of DTPA-aptamers (2), and the upper (3) and lower (4) portion of the band from lane 2 in Figure 2.9.

Figure 2.11 shows an 8% denaturing PAGE gel of the DTPA aptamers incubated with (lane 2) and without gold (lane 3). Lane 1 is a 10 bp DNA ladder. Since the standard coupling chemistry must be altered for sulfur containing modifications, the efficiency of incorporation for these types of modified nucleotides is often significantly reduced. The lower bands in the gel are aborted sequences that do not contain the DTPA modification. The band corresponding to the full length oligonucleotide containing the DTPA modified 84-mer is not present in the reaction containing gold (Lane 2). The gel also shows that the aborted sequences during DNA synthesis, which do not contain a DTPA modification, do not conjugate to the gold nanoparticle surface when it is protected with the bis phenylphosphine. This implies that our nanoparticle monolayer surface passivation strategy was successful in



Figure 2.9: Analysis of gold nanoparticles treated with or without DTPA aptamers.



Figure 2.10: Analysis of DNA-nanogold conjugates purified by agarose gel electrophoresis.

preventing the aptamers from nospecifically binding to the gold surface which would render their binding properties non-functional.

Conclusions and Future Directions



Figure 2.11: 8% Denaturing PAGE gel DTPA aptamers +/- gold.

Two different methods for functionalizing gold nanoparticles were developed. Depending on the application sought after, these strategies offer certain advantages over one another. The mixed monolayer conjugation strategy that was presented affords more control over the precise number of aptamers that can be physically conjugated to the surface. However, it also appears that discrete DNA-gold nanoparticle conjugates can be purified from agarose gel with the bis phenylphosphine/DTPA strategy and that the average number of aptamers per nanoparticle can be adjusted by incubating with a different molar ratio of DTPA terminated aptamers. Since the DTPA modification was incorporated into modified ssDNA aptamers that target the VEGF receptor complex previously by a collaborator (SomaLogic), we continued to use this modification rather than specially purchase a maleimide terminated ssDNA molecule as we had difficulty in getting the heterobifunctional crosslinking strategy to work. The gold nanoparticle-aptamer conjugates were designed to be used in cell targeting strategies and imaged in electron microscopy for the visualization of membrane bound ligands and proteins. They will also be investigated for their ability to facilitate receptor mediated uptake of gold nanoparticles based on the aptamer conjugated to the nanoparticle surface.

Chapter 3

Aptamer Nano-Gold Conjugates for Targeting the VEGF Receptor Complex

Introduction

VEGF Signaling

Vascular endothelial growth factor (VEGF) is a cytokine and specific mitogen for vascular endothelial cells. The VEGF family consists of seven members, named -A through -F, and placental growth factor (PIGF). They share a common structure of eight characteristically spaced cysteine residues in a VEGF homology domain and activate one of three VEGF receptors (-R1, R2, and R3) as either homo or heterodimer pairs [28]. VEGF receptors are receptor tyrosine kinases (RTKs) which are found embedded in the transmembrane region. RTKs initiate intracellular signaling cascades through the autophosphorylation of intracellular tyrosine residues and are endocytosed by the cell into early endosomes upon ligand activation.

The VEGF signaling pathway plays an essential role in angiogenesis, which is the process of sprouting new blood vessels from existing vessels. Embryonic development, cardiovascular development and the sprouting of new blood vessels during tumor development are all dependent on the VEGF pathway in some respect [29]. Poor patient prognosis is associated with elevated levels of VEGF in the blood of cancer patients [30]. Enhanced VEGFR2 expression as well as mutations that create constituitively active VEGFR2 receptors are also associated with oncogenesis [31]. The classic model of RTK activation is a ligand induced dimerization between two membrane associated receptors, which initiates a signaling cascade through downstream intracellular effectors. Oncogenic mutations in the VEGFR-2 receptor lead to angiogenesis through a variety of mechanisms including focal adhesion turnover, cell proliferation, cell migration, cell permeability and overall cell survival. Some of the major downstream signaling proteins that are activated due to VEGFR2 mediated signaling include DAG, IP3, Ras/Raf, MEK1/2, Atf and FAK ([29], [31]).



Figure 3.1: Different binding modes of $VEGF_{165}$ and $VEGF_{121}$.

There are also a variety of RTK co-receptors that associate with ligands bound to specific homo- or heterodimer pairs. The nature of the mechanisms controlling the formation of these complexes remains an open area of investigation [32]. VEGF-A is a secreted dimeric glycoprotein that exists as four major isoforms in humans. VEGF-A₁₂₁ and VEGF-A₁₆₅ are the most abundant isoforms secreted by the cell and regulate the formation of new blood vessels from existing vessels through activation of VEGFR-2. Signaling is augmented when VEGF-A₁₆₅ associates with heparan sulfate proteoglycans (HSPGs) and Neuropilin-1 (Nrp-

1). Some have speculated that the co-receptors enhance the strength of interaction between the heterodimer of VEGFR-1/2 complex while others argue that the co-receptors promote clustering of active dimers into lipid rafts within certain regions of the cell membrane [33].

Interestingly, $VEGF_{121}$ and $VEGF_{165}$ differ in their ability to activate VEGFR-2mediated effects on endothelial cells when Nrp-1 is present. It has been shown by previous studies that exon 7 of $VEGF_{165}$ shares a binding interface with Nrp-1 [34]. Exon 7 is lacking in $VEGF_{121}$ and it has been proposed that the increased signaling activity induced by $VEGF_{165}$ with respect to $VEGF_{121}$ is due to its potential to form clusters of dimerized receptors [35]. A schematic representation of the differential binding between VEGF isoforms, Nrp-1 and VEGFR-2 is shown in Figure 3.1. We intended to label this signaling complex with Nrp-1, VEGFA and VEGFR2 aptamers conjugated to distinct sizes of nanoparticles in a multiplexed labeling strategy.

Biofunctionalized Nanoparticles for Imaging Cells in Transmission Electron Microscopy

The transmission electron microscope has been used to image the structure and architecture of cellular components, membranes and protein complexes in their native or near native environments. Since an electron microscope uses a beam of electrons rather than a beam of light as the source of irradiation, the resolution limits it can achieve are about ten fold greater than fluorescence based methods. However, one major advantage of fluorescence microscopy is the ease by which an investigator can genetically encode a fluorescent protein as a fusion to the target protein under investigation. Since most individual proteins are irregulary shaped and are dispersed amongst a milleau of other proteins within the nucleus, cytoplasm and membrane, it is usually necessary to identify a target of interest in electron microscopy with the use of an electron dense tag so that an investigator can identify the location and movement of the protein through the cell's organelles and trafficking machinery [36]. Spherical gold nanoparticles are used in EM as recognizable, electron dense markers that identify the location of specific proteins in either whole cells or within purified, reconstituted protein complexes. In order to direct gold nanoparticles to proteins or cell-types of a specific interest, a biorecognition element is typically conjugated to the nanoparticle surface that targets a membrane bound receptor, which is overexpressed due to an abnormal phenotype. We set out to create a multiplexed labeling strategy whereby distinct components of a macromolecular complex would be labeled by distinct sized nanoparticles conjugated to VEGF, VEGFR2 and Nrp-1 aptamers, respectively. The aptamers used were slow-off rate modified aptamers (SOMAmers) developed by SomaLogic and had not been previously characterized for the location where they bind their respective protein target.

EM also enables investigators to create three dimensional topology maps of protein assemblies in order to understand the spatial relation they have to one another within the context of the cell or as a purified macromolecular complex. These types of images are crucial to understanding relationships between structure and function in biological systems. Since cells must be fixated before being imaged under an electron microscope, this technique offers only snapshots of cellular events in time. Cryofixation keeps cells in their most native environment by instantaneously freezing cells grown on a formvar coated copper grid such that a layer of vitreous ice keeps the cells in a sold, amorphous phase of water [37].

A more common alternative to cryofixation is chemical fixation whereby by a chemical crosslinking agent, such as glutaraldehyde, is added to create covalent bonds between proteins in the cell. This anchors soluble proteins to the cytoskeleton and cell membrane, which can then be stained and embedded in epoxy before being cut into thin sections, placed on a copper grid and imaged in a TEM. This method of fixation is also very technically challenging as it requires very steady coordination while sectioning a sample under a microtome, which takes much practice to become proficient at. Special thanks is given to Mary Morphew in the Boulder Lab for 3D EM of Cells as she helped guide me through the EM sample preparation process.
A549 Cell Line as a Model System for the VEGF Pathway.

The A549 cell line was initiated in 1972 from an explant of lung carcinoma that was excised from a 58 year old male patient. It has an epithelial morphology and grows as an adherent monolayer on tissue culture plates. The A549 cell line has served as a model system for VEGF signaling in peer-reviewed research reports within the field [39]. Nrp-1, VEGFR-2 and VEGFA have been shown to be expressed in the A549 cell line [40].

Fluorescence Polarization Anisotropy

Flourescence Polarization Anisotropy (FP) is a phenomena that is observed when polarized incident light excites a fluorophore that subsequently emits photons according to the fluorophore's intrinsic anisotropy, tumbling rate in solution and fluorescence lifetime. The mathematical relationship equating anisotropy (r) to the intrinsic anisotropy of a molecule (r₀), the rotational time constant (ϕ) and the fluorescence lifetime (τ) is given by $r = \frac{r_0}{1+(\tau)/(\phi)}$. The fluorescence lifetime is the amount of time it takes the fluorophore to emit a photon after being excited at its excitation maximum, which is usually on the order of a few nanoseconds. The intrinsic anisotropy of a fluorophore can be thought of as an intrinsic property of the molecule, not dependent on the tumbling rate or fluorescence lifetime but rather the amount of light the fluorophore depolarizes based on the asymmetric path the photon takes through the molecule between absorbtion and emission. The parameter that changes in a protein-ligand binding experiment is the rotational time constant. This constant is related to the tumbling or rotation that the fluorophore experiences in solution. When a small, fluorescently labeled ligand is in its unbound state, it tumbles fast and depolarizes light more quickly than when it is bound to a much larger protein whereby depolarized emission occurs to a lesser extent relative to the timescale of an FP experiment, which is on the order of nanoseconds. Figure 3.2 illustrates this concept.

A fluorometer enables an investigator to irradiate a sample containing a fluorophore or fluorophore linked molecule with polarized incident light at its excitation maximum and



Figure 3.2: The tumbling rate of a fluorescent molecule in solution measured by FPA.

measure the emitted photons using photomultiplier tube (PMT) detectors positioned in exact perpendicular planes with respect to the incoming polarized light. When using this experimental approach, anisotropy is defined as the ratio of polarized light component to the total light intensity by the equation: $r = \frac{I_{VV} - G*I_{VH}}{I_{VV} + 2*I_{VH}}$. Where the G-factor is defined as $G = \frac{I_{HV}}{I_{HH}}$ The polarized intensities are represented by I_{VV} , which is the intensity measured with excitation and emission polarizers mounted vertically, and I_{HH} , which is the intensity measured with excitation and emission polarizers mounted horizontally. The depolarized intensities are represented by I_{HV} ,, which is the intensity measured with excitation polarizer mounted horizontally and emission polarizer vertically and I_{VH} , which is the intensity measured with an excitation polarizer vertically mounted and emission polarizer horizontally mounted. A schematic representation of a fluorometer setup is shown in Figure 3.3.



Figure 3.3: Schematic representation of fluorometer setup.

FP is unique among other methods used to analyze molecular binding events because it gives a direct, nearly instantaneous measurement of a fluorescently labeled ligand's bound to free ratio. It also enables a direct view of molecular binding events in solution, allowing true equilibrium analysis with very low concentrations of fluorescently labeled molecule to a limit that is determined by the detection limit of the fluorometer. FP measurements also do not physically alter samples, which is a drawback of many other binding assays that require the physical separation of bound and unbound ligands because these processes can effect equilibrium while performing the assay. An FP experimental protocol was developed to measure K_D , k_{OFF} and k_{ON} of aptamer-protein binding events. We also used it to qualitatively investigate the VEGF-A aptmaer binding interface with VEGFR2 and heparin sulfate.

Experimental and Results

Development of FP Assay for Studying Aptamer-Protein Interactions

In FP measurements involving a fluorescent dye tethered to an aptamer, it is important not only to optimize the fluorophore but also to minimize the number of rotatable bonds by rigidifying the linkage between the aptamer and the fluorophore so that the fluorophore accurately tracks the rotation of the aptamer when in solution. In a previous report which compared the behavior of several DNA aptamer -fluorescein and -Texas Red conjugates, it was shown that overall Texas Red produced a better signal to noise ratio as a tracer in FP when used in protein IgE binding experiments [41]. The CalFluor 610 phosphoramidite (Figure 3.4) is a dT modified fluorophore that has nearly identical photophysical properties as Texas Red in terms of its fluorescence lifetime, quantum yield, excitation maximum and emission spectra. It can also be incorporated at the 5' end of a synthetic oligonucleotide during solid phase synthesis. VEGF-A, VEGFR-2 and Nrp-1 ssDNA aptamers were synthesized with a 5' terminal CalFluor610 phosphoramidite and used in the FP experiments.

Determining the Linear Detection Range of the FP Assay

Another experimental parameter to consider is the slit widths used within the polarizers at the emission and excitation source. The slit widths determine how much light makes it to the PMT detectors and therefore the overall intensity counts. However, as slit widths get larger, changes in anisotropy become more difficult to delineate as readings become more noisy. We found that 6 nm slit widths were sufficient to generate enough counts and accurately measure changes in anisotropy in our experiment.

Since the signal to noise ratio in the experiment is optimal within a certain range of



Figure 3.4: Structure of the CalFluor610 Phosphoramidite.

fluorescence emission counts detected by the PMT, it is necessary to determine the linear detection range of the fluorophore to be used in the assay so that the concentration of fluorophore linked molecules in solution can be kept as low as possible within the detection limits of the fluorometer. In order to accurately calculate the concentration of CalFluor labeled aptamers in solution via a calibration curve and to determine the linear detection range of the fluorometer, a dilution series of CalFluor 610 phosphoramidite was prepared between 1 and 100 nM. The emission spectra were collected from 605 nm to 700 nm and are shown in Figure 3.5. Figure 3.6 is a plot of each peak intensity in Figure 3.5 versus the corresponding CF610 concentration.

Using our experimental setup, the CalFluor610 phosphoramidite excitation maximum was determined to be 585 nm with an emission maximum at 607 nm. Peak emission intensities at 607 nm are plotted against concentration in Figure 19. Between 1 and 100 nM concentrations, the CalFluor 610 phosphoramidite is within the linear range of detection of the fluorometer and produced enough counts for accurate anisotropy values to be measured.



Figure 3.5: Dilution series of CF610 at 6 nm slit widths.

We found that below 1 nM, the PMT detectors did not detect enough flourescence counts to produce accurate anisotriopy measurements (> 1 X 10⁶). This is not a problem for k_{OFF} or k_{ON} measurements but would be considered an experimental limitation for aptamers with K_D binding constants in the picomolar to low nanomolar range.

Determining anisotropy values for the bound and unbound aptamer.

In order to design an FP based protein binding experiment, a measurable change in anisotropy of the fluorophore linked molecule when in its bound and unbound state must be evident. If the fluorophore is linked to a nucleic acid aptamer, the rate at which the labeled molecule tumbles should decrease significantly when it is bound to a large protein, which will depolarize less light and increase the anisotropy value. Assuming 1:1 binding, a plot of concentration vs. anisotropy should yield a MichaelisMenten curve that can be fit to linear least squares analysis to determine the K_D binding parameter, which is the concentration at which half maximal binding is observed.



Figure 3.6: Peaks from the CalFluor610 serial dilution plotted versus concentration.



Figure 3.7: Change in anisotropy of VEGF-A aptamer with addition of ligand.

The change in anisotropy was measured upon addition of a ten-fold excess of VEGF₁₆₅ protein to the VEGF-A aptamer and compared to the change associated by adding 10 uL of 1X Buffer supplemented with 1% BSA, which should not affect the anisotropy. As the fraction of labeled molecules that are bound increases, the anisotropy value increases to a maximum value that corresponds to the fluorescent molecule being 100% bound. Figure 3.7 shows that the anisotropy value for unbound CalFluor 610 labeled aptamer was determined to be 0.2455 (+/-) 0.00076 while the aptamer in the bound state was determined to be 0.2758 (+/-) 0.0013. This equates to a relative change in anisotropy of about 0.03, which is consistent with other reports that measured changes in anisotropy of similar sized native ssDNA aptamers with VEGFA [42].

After determining anisotrpy values for the unbound and fully bound state, an experimental protocol was established in order to determine the K_D of the VEGF-A and Nrp-1 aptamers by titrating increasing amounts of protein while keeping the concentration of fluorophore labeled aptamer constant. FP was also used to determine k_{OFF} of the VEGF-A aptamer by saturating the fluorescently labeled aptamer in solution, then adding excess unlabeled aptamer and measuring the anisotropy value over time, which produced an exponential decay curve that was subjected to a linear least squares analysis fit to determine $t_{1/2}$ and K_{OFF} . For k_{ON} experiments, we attempted to observe association over time as aptamers went from an unbound to fully bound state over a range of VEGFA concentrations. However, the association happened so quickly that we could not observe early data points within the experiment as we were limited to 12 second increments between measurements while the association between aptamers and their respective proteins was over within 30 – 60 seconds.

Determination of KD for VEGF-A and Nrp-1 modified ssDNA aptamers

When determining K_D , the concentration of CalFluor610 labeled ssDNA was kept as low as possible but within the linear detection range of the fluorometer at a concentration of 3.5 nM in 180 uL 1X SB18 Buffer. The aptamers were heated to 75 C for 5 minutes and allowed to cool to ambient temperature to induce thermal melting and renaturation according to SomaLogic's thermal annealing protocol. The fluorescently labeled aptamers were pipetted into a 250 uL quartz mini-cuvette and their unbound anisotropy was measured with excitation and emission polarizers set to 6 nm slit widths at 590 and 611 nm, respectively. The emission spectra was also collected in order to accurately determine the concentration of fluorophore labeled molecules in solution based on the calibration curve from the CalFluor610 serial dilution. Purified recombinant proteins (R&D Systems) were titrated into the same quartz cuvette using a 100 uL gel-loading pipette tip. Upon each addition of protein to the cuvette, the pipette was reset to 100 uL and the solution was gently pipetted up and down before measurements were taken at each data point. The protein-aptamer solutions were incubated for 15 minutes at ambient temperature to allow equilibrium to be established before anisotropy measurements were taken. Five data points were collected for each concentration measured and the average value is plotted with standard deviations at each data point. Figure 3.8 shows the results of this experiment for the VEGF-A aptamer while Figure 3.9 shows the results for the Nrp-1 Aptamer

The data were analyzed using IgorPRO software in order to determine the K_D . The fit equation f(x) = ((Bmax * [L])/(Kd + [L])) was used as the fit function. According to the curve fit, the VEGFA Trp du modified aptamer had a K_D value of 61 nM (+/-) 10 nM while the Nrp-1 Benzyl dU modified aptamer had a K_D value of 18 nM (+/-) 2.2 nM.

Determination of K_{OFF} for VEGFA modified ssDNA aptamer

The K_{OFF} assay was initially designed using a CalFluor610 labeled 50-mer Trp-dU modified VEGF-A aptamer but in the process of optimizing the assay we found that a 30-mer truncate sequence, which is the same aptamer but without 20 nt of non-functional DNA, performed much better giving us a two fold increase in the signal to noise ratio (Figure 3.10). The non structural ssDNA component may have limited the ability of the fluorophore



Figure 3.8: VEGF-A aptamer binding curve (top) and curve fit analysis (bottom)



Figure 3.9: Nrp-1 aptamer binding curve (top) and curve fit analysis (bottom)

to accurately track the aptamer's rotation in solution.

The K_{OFF} was determined for the VEGF-A aptamer by establishing the aptamer-



Figure 3.10: FP assay sensitivity comparison of VEGF 30mer (left) and VEGF 50mer (right)

protein complex in its fully bound state and then adding a 20X excess of competing unlabeled VEGF-A aptamer. A 50 nM solution of VEGF-A CalFluor labeled aptamer was first measured in its unbound state to determine the baseline value for the K_{OFF} experiment. VEGF-A protein was added to a final concentration of 500 nM in order to establish the fully bound aptamer-protein complex. A non-fluorescently labeled VEGF-A aptamer was added to a final concentration of 1 uM and the anisotropy of the solution was measured after 10 seconds and collected every 12 seconds over time wherein an exponential decay curve is observed. A first order dissociation process was assumed but without varying the concentration of unlabeled aptamer, this could not be known for sure.



Figure 3.11: Anisotropy vs. Time in K_{OFF} experiment (top) and curve fit analysis (bottom)

The data were fit using IGOR software. Figure 3.11 shows an exponential decay curve where the $t_{1/2}$ value was determined to be 2850 seconds or 47.5 minutes. This corresponds to a k_{OFF} value of 0.000240 sec ⁻¹ or 0.0146 min⁻¹. Using the experimental values for K_D and k_OFF , k_ON for the VEGF-A aptamer was calculated to be 2.4 X 10⁵ M⁻¹ min⁻¹. This value

could not be experimentally determined due to extremely fast association kinetics that could not be observed within the time resolution limits of our fluorometer which could capture a data point every eleven seconds at the fastest.

Qualitative investigation of VEGFA aptamer binding interface

The VEGFR2 extracellular domain was added to an already established VEGFA aptamer-protein complex. A subsequent increase in anisotropy shows that a second binding event occurs between most likely the VEGF protein and the extracellular domain of VEGFR2 (Figure 3.12). Conversely, the addition of 20 uM heparin sulfate to the established VEGFA aptamer-protein complex reveals that the VEGFA aptamer likely binds VEGF protein in the heparin sulfate binding domain (Figure 3.13). VEGFA Trp dU modified ssDNA aptamer does not occlude VEGFR-2 from binding VEGFA Protein but diseasociates from VEGF protein upon addition of heparin sulfate.



Figure 3.12: VEGFR-2 added after VEGF-A aptamer/protein complex established



Figure 3.13: Heparin sulfate added after VEGF-A aptamer/protein complex established

Treatment of A549 Cells with Aptamer-Nanogold Conjugates

A549 cells were grown as an adherent monolayer between 70% and 80 % confluency in F12-K media supplemented with 10% FBS. The media was aspirated off and the cells were rinsed with 1X PBS before being treated with aptamer-nanogold conjugates. The aptamer - nanogold conjugates were applied to the cells at 100 nM. A549 cells were allowed to incubate with the labeling reagent for 15 minutes at 37 C in a 5% CO_2 atmosphere before being rinsed again with 1X PBS, fixated with 2% glutaraldehyde, stained with heavy metal contrast agents and embedded in epoxy overnight. The next day, samples were cut into 85 nm cross sections on a microtome and placed in sequential order next to one another on an EM grid.

When we set out to use our aptamer nano-gold conjugates for cell labelling in EM, we intended to probe the proposed clustering of receptors that was presented in the introduction with whole cell electron microscopy. Using gold nanoparticles of defined dimensions conjugated to ssDNA aptamers which detect distinct components of this multiprotein receptor complex (Nrp-1, VEGFA₁₆₅ and VEGF-R2), we thought that imaging co-localized nanoparticles at the cell surface would not only be achievable but also reveal biologically relevant results. We soon realized that the dynamic nature of the cell membrane would make such

an endeavor quite difficult.

Instead, we noticed rapid uptake of nanoparticle-aptamer conjugates but had trouble differentiating between observations made between scrambled, non-binding sequences and aptamer functionalized nanoparticles that appeared to be bound to the cell surface. Probably the only interesting outcome worth reporting was that in some instances, we could see direct evidence of nanoparticle uptake within clathrin coated pits, which are regions of the cell membrane specialized in receptor-mediated endocytosis. We could also see evidence of clathrin coated vesicles budding off from the membrane as they transported proteins attached to nanoparticles to early endosomes. We saw an increased uptake of nanoparticles conjugated to modified aptamers that target VEGF and VEGFR2 compared to a scrambled sequence containing benzyl dUTP modifications in both clathrin coated vesicles and early endosomes.

In order to systematically address receptor mediated uptake of nanoparticles, the number of nanoparticles endocytosed or found on the surface of A549 cells per um² was determined. This was calculated by dividing the total number of nanoparticles by the cross sectional depth of the sample (85 nm) multiplied by the total length of the membrane in the thin section. A low magnification image of every cell section investigated was taken and the cell perimeter was approximated using Image J software.. Cells were labeled with 5 nm VEGFA aptamer nanogold and 10 nm VEGFR2 aptamer nanogold or with 5 nm VEGFA aptamer nanogold and 10 nm benzyl modified scrambled sequence.

A549 cells labeled with 5 nm VEGFA aptamer nanogold conjugates and 10 nm scrambled sequence nanogold conjugates showed the internalization of mostly 5 nm diameter gold nanoparticles. While some 10 nm particles are found on the cell surface and within the cytoplasm, we did not find any evidence of clathrin dependent endocytosis of 10 nm gold particles conjugated to a scrambled benzyl block sequence. Figure 3.16 illustrates that when comparing the scrambled sequence, VEGFR2 and VEGFA aptamer, 14.1%, 46.3% and 49.5% were endocytosed, respectively



Representative TEM images of A549 cells treated with 5 nm VEGF-A (red circles) and 10 nm scrambled sequence (blue circles) gold conjugates. In the top left image, 5 nm gold particles are seen in well-defined vesicles. In the top right and bottom left images, 10 nm scrambled sequence conjugates are found at the cell surface and were much less often found endocytosed. In the bottom right image, 5 nm gold particles are seen in membranes budding into a clathrin coated pit, which is a precursor to early endosomes in the internalization of activated RTK receptors.

Figure 3.14: TEM images of A549 cells treated with 5 nm gold - VEGFA aptamer and 10 nm gold scrambled sequence



Representative TEM images of A549 cells treated with 5 nm VEGF-A (red circles) and 10 nm VEGFR-2 (green circles) gold conjugates. In the top left panel, 10 nm particles are internalized in well defined endosomes (E) along with 5 nm particles. In the top right and lower left images, 10 nm particles are seen in early endosomes and were found to be internalized at a higher frequency than 10 nm particles conjugated to a scrambled sequence incubated with A549 cells over the same length of time.

Figure 3.15: TEM images of A549 cells treated with 5 nm gold - VEGFA aptamer and 10 nm gold- VEGFR2 aptamer

Conclusions and Future Directions



Figure 3.16: Number of nanoparticles per cell surface area

While we set out to label the VEGF receptor complex with a multiplexed nanoparticle labeling strategy, the experiment proved to be incredibly difficult to execute in practice. VEGFR2 conjugated gold nanoparticles were endocytosed into cells, in many instances along with 5 nm particles conjugated to VEGFA aptamer. In summary, VEGFR2 aptamers were uptaken into well defined vesicles within the cell more often than the scrambled sequence. While more evidence of receptor mediated uptake needs to be built, this is a potential indication that the VEGFA aptamer and VEGFR2 aptamers can be internalized via a receptor mediated endocytotic pathway. This pathway is the major way by which DNA, siRNA and proteins are uptaken into cells whereby they are eventually degraded in the lysosome.

There are a variety of methods that help facilitate the endosomal escape and ensure cytosolic delivery of therapeutics [43]. Strategies for efficient and specific delivery of targeted therapeutic agents in biological treatments such as protein and gene therapy remain difficult despite continuous improvements in our ability to manipulate cell behavior [44]. Several overarching limitations for developing these strategies include improving accumulation of payload delivery in the right tissue type or cell compartment, maintaining stability of the active biological component, evading the auto-immune response and releasing payload delivery in response to biological cues that maximizes the therapeutic effectiveness of the targeting molecule [43]. A potential next step in developing aptamer gold nanoparticle conjugates would be to offer a delivery mechanism that can be rendered multi-functional and direct nanoparticles to certain cellular compartments or escape into the cytoplasm after their internalization into endosomes.

As for the FP data, perhaps the most important observation was the marked improvements in the signal to noise ratio in our assay when using a VEGFA 30mer truncate compared to a 50mer that had 20 nt of non-functional DNA between the binding portion of the aptamer and the 5' fluorophore. Since FP reports the collective anisotropy of the population of fluorophore's in solution, a region where a non-functional portion of DNA adopts more than one conformation or no regular structure in the folded aptamer, it could severely limit the use of fluorophore conjugated aptamers in FP. We had difficulty in measuring the VEGFR2 aptamer altogether in our binding studies as there was a high signal to noise ratio observed when measuring the unbound and bound anisotropy. This could have been due to the fact that there was non-structural sequence between the fluorophore and aptamer binding domain. Other factors, such as the stability of the protein being measured in the binding buffer could also have contributed to noise within the experiment.

In order to determine k_{ON} rates in future experiments, greater time resolution is needed in the fluorometer setup, which was an unexpected limit to our experimental detection method. Typically, antibody/protein and native aptamer/protein interactions are not reported associate on the time scales that we observed. It is possible to achieve this greater time resolution through modification of a fluorometer to a T-format rather than an L-format and this could provide the necessary data to determine k_{ON} and k_{OFF} in order to interpolate K_D through their mathematical relationship.

Chapter 4

Imidazole Modified ssDNA Sequences that Template Gold Nanoparticles

Introduction

One of the many examples in nature where functional nanostructured materials are assembled using an organism's endogenous proteins exists in the magneatosomes of magnetotactic bacteria. The iron oxide nanoparticles that they synthesize become enveloped along the surface of the membrane and provide a sensing mechanism for the bacteria by which the bacteria can orient itself along the magnetic field lines of the earth [45]. In addition to naturally occurring biomineralizing peptides and proteins, there are a variety of *in-vitro* evolutionary methods that select for sequence specific biotemplates that can assemble nanoscale materials from precursor ions. Biomineralizing peptides and nucleic acids that are evolved through *in-vitro* evolutionary methods are an emerging area of research that offers much promise for providing new templates in the bottom-up assembly of nanostructured materials by integrating inorganic, organic and biological molecules [46], [47].

In recent years, RNA, DNA and protein sequences have been discovered that afford control over the size, shape, polymorph, and hierarchical assembly of nanoparticles [48]. These nanomaterial templating sequences have been isolated by techniques such as phagedisplay and RNA SELEX. One of the many reasons why this avenue of research is so exciting is the potential discovery of new material phases that could be achieved by selecting for sequences that not only template nanomaterials, but also exhibit a functional property that is unachievable by standard routes of synthesis [49].

A central principle of materials based *in-vitro* selections of peptides and nucleic acids has been that active sequences can be partitioned from inactive sequences by isolating, eluting and amplifying sequences bound to nanomaterials after an incubation step. Binding between a biomolecule and a nanoparticle is thought occur when sequences with high affinity for surface facets of pre-synthesized nanomaterials or sequences that template and cap the nanoparticle itself are left bound to the material after washing or purification steps remove unbound and unwanted sequences. The captured sequences are exponentially enriched and regenerated into the next round's starting population where they are taken through another iteration of selection and amplification (Figure 4.1). We set out to find imidazole modified ssDNA sequences that template gold nanoparticles from Au^{3+} precursor ions in aqueous solution and developed a partitioning method that could be applied towards similar *in-vitro* selection strategies whereby sequences that template metal nanoparticles are selected based on the difference in density between sequences bound to gold nanoparticles and unbound sequences.

One of the early caveats to RNA *in-vitro* selection was the limited number of functional groups available to nucleic acid sequence space compared to the possibilities found within peptide sequence space. Particularly, aromatic hydrophobic groups and ionizable groups with pKa values near physiological pH, which enable proteins to cooperate in certain biomolecular interactions, are lacking in the native nucleotides. However, the incorporation of modified nucleotides into SELEX protocols introduced new chemical functionalities that bestow enhanced properties upon the nucleic acid library. These groups are not only distinct to the types of functional groups found in the canonical purine and pyrimidine bases but also to the native amino acids in some instances.

RNA SELEX protocols that incorporate modified UTP derivatives into the nucleic acid library have been used for the discovery of sequences which template inorganic materials from organometallic precursors and metal cations [50]. In these selections, organometallic and



Figure 4.1: Modified ssDNA SELEX scheme for nanoparticle templating sequences.

metal cation precursors were chosen that preferentially interact with pyridal and imidazolyl modifications on the UTP. Conserved sequence motifs that effect particle morphology, size, and in some cases, composition were found to be dependent upon the specific RNA sequences used to mediate particle formation [48].

In a model proposed by Berti, et. al, nucleation processes that are triggered by nucleotides or nucleic acids begin when a precursor complex forms between a metal ion and the nucleic acid. At physiological pH, positively charged metal cations interact with negatively charged phosphate groups through simple, non-specific electrostatic attraction. Alternatively, empty orbitals of metal cations can accept electrons from basic amino and keto groups within nucleobase moieties. This leads to the coordination of metal complexes which, if positioned correctly in relation to one another in the zerovalent state, could lead to the formation of metal metal-bonds and the establishment of critical nuclei. After a critical nuclei of atoms are established, a growth phase ensues whereby individual atoms begin to deposit on the nanoparticle surface. Eventually, stablizing interactions between the biomolecule and capping ligand become more thermodynamically favorable than the addition of new atoms to the nanoparticle surface and if the conditions are right, the nanoparticles in solution will remain stable as the biotemplate remains bound to the surface [51].

Certain peptides and proteins that template zerovalent gold nanoparticles from Au^{3+} ions are thought to chelate gold atoms through the imidazole side chain on surface histidines. For example, the repeating consensus sequence AHHAHHAAD from the histidine-rich protein II of *Plasmodium falciparum* was reported to mediate the aqueous self-assembly of zerovalent gold metal clusters with the aid of a reducing agent. This same sequence was subesquently shown to mediate the assembly of gold nanowires after being immobilized on heptane dicarboxylate nanowires [11]. Due to imidazoles tendency to form coordination complexes with Au^{3+} and perhaps play a direct role in peptide mediated gold nanoparticle synthesis, imidazole modified dUTP (Figure 4.2) was the modification we chose to incorporate in our modified ssDNA SELEX sheme in order to discover sequenes that mediate the formation of gold nanoparticles from Au^{3+} . However, no examples of DNA SELEX had been shown to work before with a materials based selection.



Figure 4.2: Structure of imidazole modified dUTP.

The first materials based RNA *in-vitro* selections reported upon in the literature partitioned sequences bound to nanoparticles from unbound sequences by centrifuging RNA/metal precursor incubations through100K MWCO regenerated cellulose membranes in order to flow through unbound sequences and retain sequences bound to nanoparticles [50]. These membranes are not ideal for selections since nucleic acids can non-specifically stick to the membrane and ultimately confound the results of the selection. Approaches for isolating RNA sequences bound to iron oxide nanoparticles based on the material's susceptibility to a magnetic field were also reported where the authors selected for sequences based on a desired property of the material [52]. Selection based on a material's property is an attractive idea but not all types of nanoparticles lend themselves to this approach.

One common theme among metal nanoparticle - nucleic acid templated processes is that the density of metal nanoparticles is much greater than that of oligonucleotides. We set out to develop a rapid and robust protocol for the isolation of ssDNA sequences that template nanoparticles using ultracentrifugation through a discontinuous sucrose gradient as a means of separation. Using this method, we were able to effectively partition active sequences from inactive sequences based on the difference in sedimentation velocity experienced by the DNA sequences bound to highly dense gold nanoparticles compared to unbound, inactive DNA sequences.

Our objective was to use the selected sequences in biologically based assays where aqueous environments, non-specific gold binding substrates and high ionic strength buffers could be present. The parameters of the SELEX experiment were set so that selected sequences could work at physiological pH using sub micromolar concentrations of Au³⁺ precursor. A mild reducing agent, L-tyrosine, was included at 1 mM to facilitate reduction of Au³⁺ to Au⁰. Control experiments indicate that L-tyrosine alone at 1 mM concentrations can not stabilize colloidal gold solutions over prolonged periods of time and that the imidazole modified ss-DNA plays a direct role in templating gold nanoparticles. This is the first time a materials based emphin-vitro selection has been carried out using ssDNA as the evolving biomolecule rather than RNA. This decreases the number of enzymatic and purification steps while also utilizing a biomolecule with a longer shelf-life that is easier to chemically synthesize.

Experimental and Results

Development of SELEX Partitioning Step

In order to partition active sequences from inactive sequences, we used differential rate ultracentrifugation through a discontinuous sucrose gradient. This method of separation is commonly used in ultracentrifugation of high density particles such as metal nanoparticles and viruses. Since density determines sedimentation rate, we saw the opportunity to separate gold nanoparticles from 100mer single stranded nucleic acid sequences. Since sedimentation velocity is dependent on the density of a molecule in solution, a gradient can be designed such that the bottom layer provides a "cushion" whereby the high density particles in solution sediment into a narrow band at the interface of the discontinuous gradient. To achieve separation in general, the high density particles, which in our experiment are the ssDNA capped gold nanoparticles, should move quickly through the top layer of the discontinuous gradient and halt at the more dense layer of sucrose. Meanwhile, the low density particles, unbound inactive ssDNA, should not migrate into the top layer of sucrose.

In order to determine the sucrose layer composition as well as the optimal times, ultracentrifugation speeds and temperatures to use in the SELEX partitioning step, 5 nm diameter gold nanoparticles protected with a self assembled monolayer of triethylene glycol mercaptoundecanoic ether were used as a standard. The deep red colored colloidal gold could be visually monitored before and after the centrifugation step in order to confirm their migration to the sucrose cushion. Briefly, a 30% over 67% discontinuous sucrose gradient was poured in a Beckman SW-40 polyallomer centrifuge tube with 3.75 mL 30% sucrose layered on top of 1 mL 67% sucrose. A 200 uL sample of 10 uM of TEG-MUE protected 5 nm gold nanoparticles was applied to the top of the gradient. The tubes were loaded into

a Beckman SW 40-Ti swinging bucket rotor and spun in an ultracentrifuge at 30,000 rpm for 30 minutes, 25 C. After the ultracentrifugation step, PEG_3 alkane thiol protected 5 nm diameter gold nanoparticles sediment into a visible, relatively narrow band at the boundary of the gradient (Figure 4.3).



Figure 4.3: TEG-MUE protected gold nanoparticles sediment to the boundary of a 30% over 67% sucrose gradient.

An important experimental detail to consider upon executing the partitioning step was determining a way to collect or fractionate the desired sample from the sucrose gradient. Most often, during recovery of proteins and nucleic acids from either glycerol or sucrose based gradients, successive layers are removed from the top of the gradient carefully using manual pipetting. While this method is most often suited for the separation of purified proteins after isocyanipic ultracentrifugation, it was not suitable for our application since there could still be a small fraction of a percentage of the original ssDNA applied to the top of the gradient residually left on top as we worked our way down into the sample towards the oligonucleotide capped nanoparticles. Since only a fraction of a percentage of the starting library makes it through to the partitioning step, we avoided using the aforementioned method in our protocol. Alternatively, the bottom of the pollyallomer tube can be punctured with a needle and the sample collected through fractionation. When tested, this method proved to be cumbersome as it was difficult to control the drop size and volume of the fractions consistently in the partitioning step where it is absolutely crucial to remain consistent in the method of partitioning from round to round. A third option that we found most effective was puncturing the side of the tube with an 18G syringe needle attached to a 3 mL non-stick plastic syringe and extracting out the material above from just slightly below the interface of the original gradient where the nanoparticles had sedimented. This method provided the most robust and reproducible way to extract sequences bound to gold nanoparticles from round to round during the selection.

Before the ultracentrifugation step, a mark was placed on the side of the tube 2 mm below the interface of the discontinuous gradient to indicate exactly where to puncture the tube at the end of the separation. 200 uL of 10 uM TEG-MUE protected gold nanoparticles was layered on top of the gradient, carefully placed in a swinging bucket rotor and ultracentrifuged at 30,000 rpm for 30 minutes. After the spin, 500 uL of sample was extracted from above the interface of the gradient and buffer exchanged into 1X PBS using 100K MWCO membrane spin filters. The yield was quantified using a UV-vis spectrophotometer.

The absorbance trace of 5 nm protected gold nanoparticles at a theorhetical 100% yield was compared to the average recovery of six identical experiments whereby TEG-MUE protected gold nanoparticles were subjected to the aformentioned ultracentrifugation protocol, buffer exchanged into 1X PBS using 100K MWCO spin filters (dashed). In this way, the sample was carried through each of the manipulations that would be required for the actual partitioning step. Using the peak observed at 535 nm, we calculated a 56% recovery of TEG-MUE protected gold nanoparticles. It should be noted that some of the nanoparticles were lost to the side of the pollyallomer ultracentrifuge tube and to the syringe while extracting and transferring the nanoparticles from the gradient and to the pores of the 100K MWCO membrane.



Figure 4.4: 8% denaturing PAGE gel of 500 uL fractions from the top of the gradient.

We next tested the migration of a ssDNA 84-mer through a 30% over 67% discontinuous sucrose gradient in order to ensure that unbound oligonucleotides would remain on top of the 30% sucrose and not amplify from the region where gold nanoparticles would be extracted from. A 200 uL sample of 5 uM ssDNA (one nanomole) was loaded on top of the sucrose gradient and spun at 30K RPM for 30 minutes. Successive 250 uL fractions were removed from the top of the gradient, buffer exchanged into 1X PBS buffer using a 10K MWCO membrane and concentrated to 10 uL total volume using a speed-vac apparatus. Each successive fraction was placed on an 8% denaturing PAGE gel with the fraction closest to the top of the original gradient corresponding to lane 1 (Figure 4.4). The detection limit of the gel stain used in this experiment was about 10 - 100 ng of ssDNA.

Determining Reducing Agent Strength and Buffer Composition

After achieving a reliable means of separation to partition active sequences from in-

active sequences in the materials based *in-vitro* SELEX scheme, an appropriate buffer and reducing agent were chosen to use during incubation of an imidazole modified ssDNA 40N library with HAuCl₄ in aqueous reaction conditions. Buffer choice is an important consideration not only because of the pH and ionic strength of the reaction environment but also because the components of the buffer must not facilitate nucleation and growth of nanoparticles on their own. Because of this, common buffers that contain compounds with amino groups and heterocylcles were avoided due to the direct role these groups could potentially contribute towards nanoparticle nucleation and growth processes. We decided upon phosphate buffered saline (PBS) without magnesium or calcium because this buffer is generally amenable to biological protocols and should remain relatively inert towards the reaction. We did not include calcium or magnesium because this would also eliminate the chance of evolving sequences that incorporate these metals into nanoaprticles.

Some amino acids and common biological reducing agents have been reported to serve as stablizing and capping agents in colloidal gold synthesis protocols. We intended upon choosing one at a concentration that would facilitate the reduction of Au^{3+} to Au^0 but not compete with ssDNA as a capping agent. We chose a variety of biological reducing agents that had measured redox potentials which would indicate that it was thermodynamically favorable to reduce Au^{3+} to Atextsuperscript0 at physiological pH. We experimented with NADH, citrate, oxaloacetate, ascorbic acid and L-tyrosine at 1 mM and 10 mM concentrations in order to determine which could facilitate the reduction of Au^{3+} to its zerovalent state but not produce stabile solutions of colloidal gold on their own. To do this, we monitored the solutions absorbance in the 500 - 600 nm range to see if the reducing agent alone could stabilize colloidal gold solutions in 1X PBS supplemented with 400 uM HAuCl4.

The optical density at 550 nm is plotted over time in the various HAuCl4/reducing agent mixtures (Figure 4.5). 10 mM and 1 mM NADH quickly reduced Au^{3+} to Au^{0} as evidence of colloidal gold emerged within the first 30 minutes of incubation time. Ascorbic acid, citrate and oxaloacetate produced similar results and did not lend themselves suitable



Figure 4.5: Common biological reducing agents facilitate the reduction of Au^{3+} to Au^{0} .

for our application. We did not want to use reducing agents that would facilitate the assembly of gold nanoparticles within hours of incubation time. Ultimately, we chose L-yrosine at a 1 mM concentration because when combined with 1X PBS in 400 uM HAuCl4, we noticed the slow reduction of $Au3^{3+}$ to Au^{0} that did not result in a solution with strong absorbance peak anywhere in the 500 - 600 nm range as evidenced by a mild absorbance peak at 550 nm which plateaued over time.

Imidazole Modified ssDNA Biotemplating of Gold Nanoparticles from $HAuCl_4$

In order to generate an imidazole-modified 40 nucleotide randomized ssDNA library, we used previously described methods that utilize Deep Vent (exo-) polymerase to incorporate modified dUTP during primer extension of a randomized antisense ssDNA template library immobilized on streptavidin agarose [20]. Deep Vent (exo-) polymerase is able to incorporate this modified dUTP with high efficiency and generate full length product at greater than 95% yield. A detailed experimental protocol of how this modified ssDNA library was generated

can be found in section 5.3.2.

After generating one nanomole of imidazole modified ssDNA library for the SELEX experiment, 350 picomoles of ssDNA library containing a 40 nt random region was dissolved in 0.1 M Phosphate Buffer, pH 7.4 with 1 mM L-Tyrosine to a final volume of 1 mL in a DNAse/RNAse free, non-stick eppendorf tube. An identical reaction without L-tyrosine was also prepared in order to see if imidazole modified ssDNA could facilitate the production of gold nanoparticles without a reducing agent. The solution was heated to 75 C for 2 minutes to induce thermal melting of the ssDNA molecules in solution and the the reaction mixtures were allowed to slowly cool to ambient temperature, 0.5 uL of 0.8 M aqueous gold (III) chloride was added to a final concentration of 400 uM. As a negative control, a reaction mixture that contained no ssDNA but all other components was prepared. The reaction mixtures were vortexed briefly and incubated at room temperature for 17 hours. Figure 4.6 shows that the reaction containing imidazole modified ssDNA library (blue) exhibits a strong absorbance peak at 535 nm, indicating the presence of gold nanoparticles. In comparison, the reaction without ssDNA (green dash) absorbs weakly throught the 500 - 600 nm range without a comparitively defined peak

SELEX Partitioning Step

After 17 hours of incubation, the reaction mixture containing gold nanoparticles and imidazole modified ssDNA library was layered on a 30% over 67% discontinuous sucrose gradient and spun at 30K rpm for 30 minutes, 25 C. A sample containing the same volume and concentration of imidazole modified ssDNA library without Au³⁺ ions was run opposite of the reaction incubation to ensure that unbound ssDNA sequences would not PCR amplify from the interface of the gradient under identical ultracentrifugal forces.

Gold nanoparticles that sedimented to the boundry of the discontinuous gradient were collected by puncturing the side of the tube with an 18G syringe needle. 500 uL of sample was drawn out from 2 mm below the original interface of the gradient. The extracted sample



Figure 4.6: Round 0 imidazole modified DNA library(blue) vs. buffer(green).

was concentrated on a 100K MWCO membrane and buffer exchanged into 1X PBS to a final volume of 75 uL. In order to amplify sequences bound to gold nanoparticles, PCR reactions containing a 3' biotinylated antisense primer were prepared in triplicate with 15 uL of the final 75 uL sample volume derived from the boundary of the sucrose gradient. 50 uL PCR reactions containing NEB HotStart Taq polymerase and all other PCR reaction components was prepared. A 5 uL aliquot of the combined PCR reactions was run on an 8% denaturing PAGE gel to ensure that the PCR product was the proper length and not a result of primer dimer. The incorporation of biotin to the antisense strand was also confirmed by using streptavidin to shift the band into a higher molecular weight complex.

The PCR amplified, biotinylated dsDNA was captured on 30 uL of streptavidin-agarose resin (50/50 slurry) and the native sense strand was eluted from the beads using 20 mM NaOH. The streptavidin-agarose beads were washed five times with 1X PBS buffer before being resuspended in 1X SQ10 buffer. Imidazole modified ssDNA was then enzymatically synthesized by performing a primer extension reaction on the captured biotinylated antisense

strand using Deep Vent (exo-) polymerase. The modified ssDNA was eluted from streptavidin agarose with 20 mM NaOH and the pH of the eluant containing ssDNA was neutralized with 1/4 volume 80 mM HCl. Before entering the next round of selection, the length of the modified ssDNA was determined using an 8% denaturing PAGE gel in order to ensusre the synthesized strand was of proper length (Figure 4.8, right panel). This selection cycle was repeated for 8 rounds while sequentially lowering incubation times and concentrations of gold precuror ions (Figure 4.7)

Round	[HAuCl4]	[ssDNA]	Time
1	400 <u>uM</u>	350 <u>nM</u>	17 <u>hr</u>
2	400 <u>uM</u>	200 <u>nM</u>	13 <u>hr</u>
3	400 <u>uM</u>	200 <u>nM</u>	6 <u>hr</u>
4	400 <u>uM</u>	200 <u>nM</u>	3 <u>hr</u>
5	40 <u>uM</u>	150 <u>nM</u>	1 <u>hr</u>
6	40 <u>uM</u>	150 <u>nM</u>	45 min
7	40 <u>uM</u>	100 <u>nM</u>	30 min
8	40 <u>uM</u>	100 <u>nM</u>	30 min

Figure 4.7: Conditions of SELEX experiment in each round.

Figure 4.8 (left panel) shows the PCR amplification results from round 1. The group of three traces with an inflection point at cycle 16 were identical aliquots of a reaction incubation with imidazole modified ssDNA library, 400 uM HAuCl4 and 1 mM L-Tyrosine. The group of three traces that did not cross the threshold was from a sample containing imidazole modified ssDNA library and 1 mM L-tyrosine but not HAuCl₄. Lane 1% of the 8 denaturing gel is a 10 bp ladder with the lowest band corresponding to 20 bp, the two bands in land 2 contains the anti-sense biotinylated strand of the dsDNA which runs higher than the sense, non-biotinylated strand in an 8% denaturing PAGE gel. Addition of streptavidin to the sample in lane 2 shifts only the biotinylated strand into a higher MW complex with



Figure 4.8: PCR trace of +/- Au Samples (left), 8% denaturing PAGE gel of PCR reaction (right).

one, two, three or four streptavidin subunits attached. The resultant biotinylated dsDNA from the PCR reactions containing samples from the HAuCl4, 1 mM L-tyrosine reactions were pooled, captured on streptavidin Ultra-Link Resin (Pierce) and the non-biotinylated, native sense strand was eluted from the beads using 20 mM NaOH. A primer extension reaction was prepared to generate round 2 imidazole modified ssDNA library.

Reactions containing 350 nM Rd. 0 imidazole modified ssDNA, 1 mM L-tyrosine and 400 uM HAuCl4 turned visibly red within 6 hours of incubation time. A broad absorbance trace in the 500-650 nm range with a peak at 540 nm was observed in Figure 4.6, indicating the presence of nanometer sized gold particles. Reaction mixtures without ssDNA began to turn purple with a black precipitate, indicating the slow reduction of Au³⁺ to macroscopic gold precipitate. Reactions with imidazole modified ssDNA ultimately produced solutions of a deep red intensity and were stable over time with no precipitate seen after weeks of incubation at room temperature. Samples without ssDNA turned colorless and had a visible black precipitate after 48 hours, indicating the presence of bulk gold.

After eight rounds of selection and amplification, the round 8 evolved pool was sent



Figure 4.9: Samples with and without ssDNA after 6 hours.

for sequencing and the data were analyzed with Daughter of Sequence Alignment (DOSA) software (SomaLogic). A variety of conserved 5 nt and 6 nt motifs are found throughout 7 families within which many of those same conserved motifs present themselves (Figure 4.10). This could mean that some of these 5 - 6 nt motifs have an affinity for gold ions or for certain surface facets of a growing gold nanoparticle surface. The conserved n-mers are arbitrarily color coded in the figure.

5 uL of sample from the reaction mixtures used in round 0 and round 8 of the selection were prepared for TEM analysis along with a no DNA control. To prepare samples for TEM, 5 uL of the nanoparticle containing solution was drop cast onto a carbon coated copper (400 mesh) TEM grid (Ted Pella) and allowed to dry under ambient conditions. Characterization of the morphology of the ssDNA-AuNP conjugates was performed on a Phillips CM10 bright field TEM. The diameter of gold nanoparticles that were imaged by TEM were measured using the ImageJ software measurement tool. Grids prepared with gold nanoparticles templated by round 8 and round 0 imidazole modified DNA were covered in material. Gold noparticles were less abundant in the no DNA control and were larger in size by comparison with edges that were less defined.

We found that imidazole modified ssDNA sequences are capable of templating gold nanoparticles from $HAuCl_4$ and that conserved sequence motifs can be isolated through iter-
Family	1	Family	5
		>\$44	CCTACCACAACCTGAGACGGGTGGCTCAACCCCCTACAAT
>S81	CAAGAACACCTCCAGCTAACCCA <mark>AGCCC</mark> CTT <mark>CCACC</mark> GATG	>586	GATGATTGGGGGGGCGCGCTGCTAACCTGA TGGCCAAGGAAC
>S78	ACCCCACGATACTCCAAAAAGCCCGTAGTATGCCACCGGTC	>\$76	AGCACCTGAGGGACACCACATCAACCCTAGGGCTCCATC
>S12	CTAGGACCGAGACTA <mark>AGCCC</mark> ACCATG <mark>ACCCC</mark> GGGC <mark>CCAAC</mark>	>\$85	GCACCTGAGGGACACCCACATCAACCCTAGGGCTCCATC
>S9	CAGATAAAGCCCGCTCCAGGAGAAAAGGCAGTAACCCTAC	>566	TTAACATCATGGTAACCTCCCAGCCGGCCATATGGCAGC
>S80	TCACATG <mark>AGCCC</mark> TTG <mark>CCCAAT</mark> AAGCCGG <mark>AACCG</mark> CCCCCTT	>565	CCCTTACATGACCCAACGAGAAAGCCCGGCACACCTC
>S73	GAATCACAAA <mark>AGCCC</mark> GACCATGGCCCCATCAACGAAACCA	>537	CAAGCCGACACCCACGCCCACGGTGGACATCAC
>S11	TTACCCACCCTGGCACGCCGG <mark>AGCCC</mark> GACCTGC <mark>CCAAC</mark> AA	>577	TAAACCTTAGGGGACCAGACCAAACACCCACACCTCC
>S25	GCACGG <mark>AGCCC</mark> GCTCAACAGCAGCTCACACAGCCCGAACA		
>S42	TCAGCCCTAACCCGCACTGGAGGTCATCAGCCCTGGTCCG	Family	6
>\$55	CGGACCGCCTCCAAGCCCACACGACGAAGCCCCCTACGCA	>\$75	ACAACTANNATGCGGCTNCNACCCAAACCACCCCATCCG
>S10	CCCTCACGCA <mark>CAAAC</mark> CGAAAACA <mark>AGCCC</mark> ATAGACGCGACG	>\$35	CACAGAGAGGCAGCCAAATGTGAGCGCAAACCCGCCGCTA
		>\$30	ACCTCCAGCCGGGTGCAACTAGCCA CAAACGAACCAGG
		>529	ATGAAACCCGAAGAACGGAAACCATCAGCCTGCCACAAAC
Family	2	>\$58	TCCGCCCGACGCCTAGCCACACCACAAACCACGGCCCACA
		>\$17	GCAGAATGCAGACCGACGCCAAACCGACACCCCTCACACA
>S3	GCAGGGCAGGAGAAAGTCGGCCT <mark>AACCG</mark> TGACCGAGGAAC		
>S28	GCCCAGGAGGAGTGGAAAGTTGCAAGATCATCAACCOCCA		
>S43	TCAGCGAGTA <u>CGGAC</u> AGACTA <mark>CCACC</mark> TCTCAG <mark>AACCG</mark> CAA	Family	7
>S24	TCATACGGGCGCGAAACAAGGGAGGCTCCACGG <mark>AACCG</mark>	>\$45	CCGACCCAGCCACAACTAACTTCCGGACCCGGGATTGGGA
>\$32	TGAACCAGACGG CCACCGTTAACCGCCAGGAAATCGACCC	>\$41	ACCCACACCGGACATAGGGATGCGAACGCACAGCCAAGCC
>S56	CTAAGCCAG <mark>ACCCC</mark> TA <mark>AACCC</mark> CGAATCCTGGAATCACACC	>\$27	CCGGACCTACTACAAATGGGCAGACGACCCGCATCCATGA
>\$38	CCTTGCAACAGGGAATTAGACCC <mark>AACCG</mark> GCCCCGACACCA	>\$93	ACGATCACCAGAACCTCACAGAATCCCGGAC AGACAAC
		>\$94	NNNNGATAAGCGCACCCCCACCGGCCAAGATCACTCCCTG
Family	3	>\$22	TACCGGATAAGCGCACGGGAGCGGACAAGATCACTCCCTG
	CT CC C 2 T 2 C 2 C 2 C 2 C 2 C 2 C 2 C		
2071	ACACCCCCTCCCCCCCCCCCCCCCCCCCCCCCCCCCCC		
>525	TNINTA A CATA A AGCCA ACCOMACTOR CACCACC	Ornhan	2
>2390		SS7	CGTGTCCNAGCCGGAGCCGGAGCACATCACCCGGGGGCCT
2009	ACCOCCTACCALCOCCTACCALCOCCTACCALCOCCTACCALCOCCTACCALCOCTACCACCALCOCTACCACCALCOCTACCALCOCTACCALCOCTACCACTACACACTACACACTACACT	>\$19	CCTGTTAGAGAATCAAGAAGACCCCGGGCTAAGATCGC
2001	ARGEGGIAGEARGACGACCACCECCIGACCIGACCIGA	>\$20	A CACCA COT CACCA COCCACA TA CACCA A COCCACACA COC
>210	NNNNNTNNGCAGACCAGGCCAACGIIAGACAGIICA	>520	ANDAGGACGICAGGAGGCGIGGAIACAGCAAGGGGGGGACI
2000	TGTTCAACAACCACCCCCCCCCCCCCCCCCCCCCCCCCC	>534	ACTACGGGGCGTCGTGAATCCGATCGGCCATAATGCTCGG
2312	IBITCANCACOOCCODACCA BBCCABBIBCCCARCCACB	>563	CTACCETCESCAGTEAGECCEATCEATAACCCTCTEEAT
Famile	4	>800	TCATAAACCATGCCATGCCGGCATAGAGCCAGGGGTTAA
ramiry	1	2002	ICATARACGCATGCCATGCGGGGGATAGAGCGAGGGGGTTAA
>S2	CTACGATCGCCAAAATAGAGCAT <mark>ACCCC</mark> AGGACGGGACGC		
>\$79	CATTCAGGATAGAGAGCGCG ACCCC AGCTGGCCCGGGAGA		
>\$96	GGNNTACCTCCCAGATCCCTGCACACATCACCCGACAGT		
>\$95	CCCTAGAGTGACGGGGGCCTACCCCCCGACGGCCAAAAGAC		
>\$51	AGCCACGAAATGGGTAGACCCCGATGGTGCCCTCTATCGA		
>\$52	AAGTTACAGACAAACAACCCGCCAAACCCCCCCAGCTAA		
>S21	ACTGTCAAGGCAGGCACCCCCCCCCCCCCCCCCCCCCCC		
>S70	CCCTGTCACAATGCTGCGGCCCGTCGACCAACCCCCCAAC		
>\$46	TTACAACCCCAAGGCTAGCGACCAGCAAACCCCGCCGGC		

Figure 4.10: Consensus sequences grouped into families amongst evolved round 8 pool.

ative cycles of selection and amplification. UV-vis spectroscopy of Rd. 0, Rd. 8 and no DNA control indicates that the evolved pool produces gold nanoparticles in greater abundance that are more narrowly dispersed in solution, smaller in diameter compared to an HAuCl₄ solution treated with the reducing agent alone or the Rd. 0 starting material. It should also be noted that in this experiment, the reaction volume was one-fifth and the concentration of ssDNA was nearly four fold lower than that of the Rd. 0 incubation. TEM analysis concurs with UV-vis spectroscopy revealing that the evolved pool produces gold nanoparticles with an average diameter of 8.5 nm (+/-) 2.2 nm compared to the Rd. 0 library which produced



Figure 4.11: TEM images of round 8 imidazole modified ssDNA (A), round 0 imidizole modified ssDNA library (B), and no ssDNA (C).

gold nanoparticles with an average diameter of 12.0 nm (+/-) 4.2 nm.



Figure 4.12: Size distribution of gold nanoparticles amongst rd.8 (red), rd. 0 (blue) and no DNA (black).

Conclusions and Future Directions

Active sequences that remained bound to gold nanoparticles were partitioned from



Figure 4.13: Absorbance spectra comparing no DNA (red), 100 nM rd. 0 DNA (green), 100 nM rd.8 DNA (blue). A picture of the cuvettes used in the measurement are shown on the right.

inactive sequences using differential rate ultracentrifugation through a discontinuous sucrose gradient and PCR amplified after each partitioning step. This method should also work as a partitioning step with similar nucleic acid based materials selection experiments. Before this selection was started, a native RNA based iron oxide selection was explored and we found that iron oxide nanoparticles could likewise be separated from RNA molecules of similar size to the DNA molecules used in this selection. We also explored the use of Ag^+ ions in an imidazole modified ssDNA selection scheme but since Ag^+ readily precipitates with phosphate buffers and chlorides, it was difficult to render silver amenable to our particular approach.

Our results indicate that ssDNA can serve as both a general template and a sequence specific templatate for the assembly of gold nanoparticles from gold precursor ions. We show that SELEX can be used to find DNA sequece motifs that assemble nanoparticles from Au³⁺ which was kept at significantly lower concentrations compared to methods which employ non-specific sequences. Sequences that can work with lower concentrations of precursor are advantageous for several reasons. When considering their use in biological applications, the nonspecific precipitation of gold nanoparticles should be less prevalent in assays containing complex mixtures of biomolecules. Towards energy applications, the use of less metal precuror offers huge savings in potential scale-up costs of these processes. The evolved motifs found in this selection could potentially be used as an identification tag in electron microscopy or an optical readout in a biosensor based assay if used as a fusion sequence to a protein binding aptamer whereby the formation of gold nanoparticles from Au³⁺ ions is exclusively templated by the evolved sequence. One could also imagine using these sequences as a fusion to specifically place gold nanoparticles on nanoengineered structures based on established techniques within the field such as DNA hybridization.

However, further optimization of the selected sequences needs to be complete to achieve these goals. When isolate sequences were tested, we saw evidence that concurred with previous characterizations of RNA materials SELEX outcomes which indicated that sequences work together to effect the outcome of particle crystallinity and morphology. Based on these observations, there is a high degree of probability that a coevolution of sequences will occur during materials based selections. This makes deconvolution of the evolved pool to find the best isolate towards a desired application difficult. Determining how one squence might effect another sequence or how combinations of sequence motifs that arose from the selection can be combined in such a way that resembles the evolved libary is a huge challenge without an assay that is both reliable and fast. One advantage to working with gold nanoparticles is that they are easily optically traceable. In order to investigate the use of several isolates needed to recapitulate the results of the round 8 material, a multiwell plate can be set up to test the combinatorial possibilities of isolate sequences and their ability to mediate the formation of colloidal gold from Au precursors.

Chapter 5

Materials and Methods

Surface Modification of Gold Nanoparticles with Modified ssDNA Aptamers

Materials: Gold nanoparticles (5 nm, 10 nm diameter) with less than 15% covariance were purchased from Ted Pella as a citrate stabilized solution. Bis(p-sulfanatophenyl) phenylphospine dihydrate dipotassium salt, 1, 8 octane dithiol (99%), triethyl mercaptoundecanoic ether (95%) and tetramethylethylenediamine (TEMED) were purchased from Sigma-Aldrich. Ammonium persulfate was purchased from Fisher Scientific. AlexaFluor₄₈₈ C5 malaimide, 10X Tris-borate-EDTA (TBE) pH 8.3 and high purity agarose was purchased from Invitrogen. Milli-Q H₂O (Millipore) was used in all experiments.

Methods:

5.1.1 Gold Nanoparticle Surface Passivation with TEG-MUE and 1,8 ODT

Ten 1 mL aliquots of citrate stabilized gold nanoparticles were placed into 1.5 mL non-stick eppendorf tubes and spun at 13K RPM for 30 minutes to concentrate the colloidal gold nanoparticles into a concentrated pellet. The supernatant from the citrate stabilized nanoparticles was removed and pellets of colloidal gold were collected into a final working volume of 500 uL. The optical density reading of a 1:50 dilution at 520 nm was measured and concentration of the gold nanoparticle stock solution was determined to be 1.05 uM. 1, 8 octane dithiol (99%) and triethylene glycol mercaptoundecanioc acid (95%) were diluted in 80% ethanol to a final concentration of 1 mM in 2 mL total volume in separate15 mL conical tubes. 16 ul of 1.05 uM citrate stabilized colloidal gold was resuspended in 209 uL

of 40% ethanol to a final volume of 225 uL. The thiolated ligands were added together in the following amounts to create a thiolated ligand mixture with increasing amounts of 1, 8 ODT:

Percent 1, 8 ODT	0%	1%	2.5%	5%	10%
uL 1, 8 ODT (1 mM)	0	2.25	5.62	11.25	22.5
uL TEG-MUE (1 mM)	225	222.75	219.4	213.75	202.5

225 uL of thiolated ligand mixture were combined with an equal volume of gold nanoparticles in 40% ethanol. The reactions were vortexed briefly every 30 minutes for 3 hours and were incubated overnight at room temperature. It should be noted that it was critical to add the triethylene glycol 11-mercaptoundecanoic acid first or as a mixture with 1,8 ODT as adding 1, 8 ODT first resulted in immediate precipitation of the gold nanoparticles. After overnight incubation, the gold nanoparticles were centrifuged at 13K RPM for 30 minutes to concentrate the nanoparticles into a 50uL pellet. Excess ethanol and thiolated ligand mixture in the supernatant was removed and discarded. The process of resuspending the nanoparticles in 1 mL Milli-Q H20 followed by centrifugation at 13K RPM for 30 minutes was repeated five times to bring the ethanol concentration below 1%. The mixed self assembled monolayer protected nanoparticles in Milli-Q H20 were used directly in the maleimide conjugation and agarose gel electrophoresis experiments.

5.1.2 Gold Nanoparticle Surface Passivation with bis (p-sulfanato) bisphosphine

Gold nanoparticles of discrete sizes (5 nm, 10 nm) were purchased from Ted Pella as a citrate stabilized solution. An adaptation of the protocol from Taton, et al. *Current protocols in Nucleic Acid Chemistry* Chapter 12.2 was used. 10 mL of citrate stabilized gold nanoparticles (Ted Pella) was pipetted into a 15 mL conical tube. 2 mg of bis (p-sulfanato) bisphosphine was added to the solution and the conical tube was placed on an orbital rocker overnight. The anionic phosphine molecules passivate gold nanoparticles as a monolayer but bis (p-sulfanato) bisphosphine protected gold nanoparticles are not stable at temperatures over 60 degrees celcius or at salt concentrations above 1 M. After overnight incubation, solid NaCl was added to precipitate gold nanoparticles. The color of the solution changed from red to light purple, indicating gold precipitate had formed. The particles were centrifuged at 1500X g for 15 minutes. The supernatant was discarded and particles were resuspended in 1 mL of 0.5 mM phosphine. The nanoparticles were precipitated again with solid NaCl, spun at 1500 x g for 15 minutes. The supernatant was discarded and the particles were resuspended in 1 m L of 0.5 mM bis phosphine one last time. 110 uL of 5X TBE was added to a 0.5X final TBE concentration. The nanoparticle solution was quantified using its optical density reading at 520 nm. A 0.9X molar ratio of DTPA2 Aptamers was added along with NaCl to a final concentration of .1 M. The mixture sat overnight on an orbital shaker for 16 hours.

5.1.3 Conjugation of AlexaFlour488 Maleimide to TEG-MUE/1, 8 Octane dithiol Protected Gold Nanoparticles

Alexa Fluor₄₈₈ C5 maleimide was purchased from Invitrogen as a 4 mM stock solution and was stored at -20 C. A 1:500 dilution of the stock solution was prepared in Milli-Q H20 as an 8 uM solution. After the gold nanoparticles prepared in the mixed monolayer reactions were resuspended in Milli-Q H20, 100 uL of TEG-MUE/1, 8 ODT protected gold nanoparticles were mixed with an equal volume of 8 uM AlexaFluor₄₈₈ C5 maleimide. The reactions sat at room temparture for 90 minutes. After 90 minutes, the reactions were spun at 13K RPM through a 100K MWCO membrane down to the void volume of 60 uL and resuspended in 300 uL Milli-Q H20, pH 7.0 (repeated 8 X). A 1:20 dilution of the reaction mixture in pH 7.0 H20 was placed in a mini-quartz cuvette. The water Raman was measured in the fluorometer at an excitation wavelength of 488 nm and the emission spectra from 508 - 650 nm was measured. Likewise, each reaction mixture was excited at 488 nm and the emission spectra from 508 to 650 nm was collected. The concentration of gold nanoparticles in the reaction mixtres was measured in a UV-vis spectrometer at 520 nm. The water Raman was background subtracted from the emission spectra and the spectra were normalized to one another based on gold nanoparticle concentration.

5.1.4 Agarose Gel Electrophoresis

0.85 g of high purity, molecular biology grade agarose was added to 50 mL 0.5 X TBE and heated in a microwave for 45 seconds to dissolve all of the agarose until the solution was just about boiling. The solution was allowed to cool to 65 C before being poured in an agarose gel casting apparatus where it was allowed to polymerize for one hour at room temperature. 0.5X TBE that had been freshly prepared was used as a running buffer. 10 uL of TEG-MUE/1, 8 ODT protected gold nanoparticles were loaded in each lane with decreasing concentrations of 1, 8 ODT running from left to right. The gel was run at 100 V for 2 hours, removed from the running buffer and imaged directly on an EPSON scanner.

5.1.5 Deaturing PAGE

In oder to make an 8% denaturing PAGE gel stock, 100 mL of 19:1 acrylamide/bis solution was added to 240 g of urea and 50 mL 10X TBE. Milli-Q H20 was added to bring the total volume to 500 mL and the solution was placed on a magnetic stir plate over night with a stir bar. 50 mL of denaturing PAGE gel stock was combined with 500 uL 10% ammonium persulfate and 25 uL TEMED in a plastic syringe before being cast in a 16 cm X 20 cm X 5 mm gel plate. The denaturing gel was allowed to polymerize for 2 hours before placing the gel in an electrophoresis chamber. The gel was run at 350 V for 45 minutes, removed from running buffer, stained with 1X SYBR Green nucleic acid gel stain and placed face down on a gel scanner (FUJI) equipped with a laser that can detect SYBR Green stained nucleic acids upon intercalation with the DNA backbone.

Aptamer Nano-Gold Conjugates for Targeting the VEGF Receptor Complex

Materials:Lyophylized VEGF165, and the extracellular domain of VEGFR2 and Nrp-1 were purchased from R&D Systems. 2 ug of lyophilized protein was suspended in 20 uL of 1X PBS, 0.1% BSA and split into 2 uL aliquots in DNA/RNAse free eppendorf tubes. The

A549 cell line and F12-K Media were obtained from the ATCC. Sterile tissue culture plates (35 mm, 100 mm) were obtained from Fisher Scientific. Trypsin/EDTA and Fetal Bovine Serum (FBS) was purchased from Invitrogen. CalFluor610 labeled aptamers were obtained from SomaLogic.

Methods:

5.2.1 Flourescence Anisotropy Assay Protocol

Determination of K_D

Stock solutions of Cal-Fluor610 labeled aptamers were diluted in 1X SB18 buffer to a final concentration of 3.5 nM in a non-stick, RNAse/DNAse free eppendorf tube. The solutions were heated to 75 C for 5 minutes and allowed to cool to room temperature before being pipetted into a 250 uL quartz minicuvette with a gel loading pipette tip. An emission spectra of the CalFluor610 labeled aptamers was taken in order to determine the exact concentration of fluor labeled aptamers in solution based on the CalFluor610 calibration curve. The unbound anisotropy value was measured five times and reported as an average, plus or minus one standard deviation. 10 uM stock solutions of reconstituted, lyophilized proteins (VEGFA, Nrp1) were added to the quartz mini-cuvette with a gel loading pipette tip, the solutions were gently mixed by pipetting up and down, allowed to reach equilibrium at room temperature for 15 minutes before being placed in the lab fluorometer (Horiba Jovin-Yvon). The successive concentrations used in the VEGF binding experiment were 7 nM, 14 nM, 29 nM, 58 nM, 115 nM and 230 nM while data points for the Nrp-1 binding experiment were taken at 14 nM, 41 nM, 67 nM, 92 nM, 104 nM and 150 nM. The anisotropy at each point was measured five times and the average plus or minus one standard deviation was calculated at each point.

Determination of K_{OFF}

Stock solutions of Cal-Fluor610 labeled VEGFA 30-mer aptamers were diluted in 1X SB18 buffer to a final concentration of 50 nM and volume of 180 uL in non-stick, RNAse/DNAse free eppendorf tubes. The solutions were heated to 75 C for 5 minutes and allowed to cool to room temperature before being pipetted into a 250 uL quartz minicuvette with a gel loading pipette tip. The unbound anisotropy was measured five times and averaged. 10 uL VEGFA protein was added to a final concentration of 500 nM from a 10 uM stock solution. After the fully bound aptamer-protein complex was established, 10 uL of unlabeled VEGFA 30mer from a 5 uM stock solution was added and the anisotropy was measured over time.

5.2.2 A549 Cell culture

All manipulations of cells and cell culture media were carried out in a sterile BSL-2 biosafety cabinet. A cryopreserved vial containing A549 cells in 1 mL of cryoprotectant media was recieved from the ATCC on dry ice. The vial was thawed in a 37 C water bath for 2 - 3 minutes with occasional agitation. While the cells in the vial thawed, 55 mL of Fetal Bovine Serum (Sigma Aldrich) was added to 500 mL F-12K Medium (ATCC) to make a complete growth medium supplemented with 10% FBS. The thawed cell suspension containing A549 cells was added to 9 mL of complete growth medium in a 15 mL Falcon screw cap tube. The 10 mL of cell suspension was centrifuged at 175 x g for five minutes to remove the cryoprotectant (DMSO) and pellet the cells. The pelleted cells were taken up in approximately 10 mL of complete growth medium and pipetted in equal volume into two 35 mm tissue culture dishes.

The two 35 mm tissue culture dishes containing 5 mL of the A549 cells resuspended in complete growth medium were incubated at 37 C in a 5% CO2 atmosphere. Changes of the complete growth medium were carried out every 18 - 24 hours. After 3 days of incubation, the A549 cells had grown to nearly 80% confluency and were ready to split. The growth medium was removed with a sterile glass pasteur pippette tip connected to a vaccuum apparatus. 2 mL of 1x PBS was added to rinse the cells and was aspirated off. 2 mL of trypsin was added to release the adherent cells from the tissue culture plate. The plate was incubated at 37 C for 5 minutes, the cells were investigated under a light microscope to ensure that the cells

had disloged from the plate. 2 mL of serum supplemented media was added and the cells were pipetted up and down to ensure even dispersion of the cell suspension.

The cells were kept at 37 C while 20 ul of the cell suspension was placed on a hemocytometer. The total number of cells per milliliter were approximated to be 400,000. Four milliliters of cell suspension of this density equates to 1.6 million. The cells were split into aliquots of approximately 100,000 cells per1 mL in F-12K media with 5% (v/v) DMSO and stored on liquid nitrogen at -80 for future experiments.

5.2.3 Growth of A549 Cells for Labeling Experiements

A cryopreserved vial of approximately 100,000 A549 cells was thanked in a 37 C water bath, the cells were removed from the cryoprotectant and resuspended in complete growth medium. A549 cells were grown on carbon coated sapphire discs that were adhered to the bottom of wells with Matrigel within a 24 well tissue culture wells. Cells grewan adherent monolayer between 70% and 80% confluency in F12-K media supplemented with 10% FBS. The media was aspirated off and the cells were rinsed with 1X PBS before being treated with aptamer - nanogold conjugates. The aptamer - nanogold conjugates were applied to the cells at 100 nM. A549 cells were allowed to incubate with gold nanoparticles for 15 minutes at 37 C in a 5% CO_2 atmosphere before being rinsed again with 1X PBS. After the labeling and washing steps, cells were fixated using a 2% glutaraldehyde fixation protocol to prepare thin sections for TEM analysis. The 1X PBS was aspirated off and 1 mL of 2% glutaraldehyde in 50 mM cacodylate buffer was added for 15 minutes. The cells were rinsed in 50 mM cacodylate buffer 5 times in a chemical hood. OsO4 (1%) in 50 mM cacadylate was added as a heavy metal contrast agent. Next, cells were embedded in epoxy over night. The next day, samples were cut into 85 nm cross sections on a microtome and placed in sequential order next to one another on an EM grid so that sequential slices of thin sections could be investigated.

Imidazole Modified ssDNA Sequences that Template Gold Nanoparticles

Materials: Triethylene glycol mono-11-mercaptoundecyl ether (95%), molecular biology grade sucrose, L-Tyrosine (99%), and gold (III) chloride hydrate (99.999%) were purchased from Sigma-Aldrich. Citrate stabilized gold nanoparticles were purchased from TedPella. Taq polymerase, dATP, dGTP, and dCTP were purchased from Invitrogen. Deep Vent (exo-) polymerase was purchased from New England Biolabs. Non-stick, DNAse, RNAse-free eppendorf tubes were purchased from BioExpress (Boulder, CO). 10K, 30K and 100K MWCO spin filters were purchased from Millipore. Pollyallomer ultracentrifuge tubes were purchased from Beckman-Coultier. All standard reagents and columns for DNA synthesis were purchased from Glen Research. 5' and biotinylated 3' primers were obtained from IDT. Milli-Q water was used in all experiments.

Methods:

5.3.1 ssDNA library Design, Synthesis and Purification

The ssDNA library used in the gold nanoparticle selection scheme was constructed as follows:

79-mer ssDNA library 5'-ATA TAT ATA CCG AGC ACT GAG TTT GCC - 40N -GCG AAA CGA CAA GAA GAC AAA AAA AA-3', 5' Forward Primer - ATA TAT ACC GAG CAC TGA GTT TGC C, 3' Reverse Biotinylated Primer - Biotin - TTT TTT TTG TCT TCT TGT CGT TTC GC.

The 40N random ssDNA library flanked by constant priming regions was synthesized on the lab's ABI-394 synnthesizer. DNA phosphoramidites were diluted in dry acetonitrile in an argon purged glovebox and placed on the lab ABI-394 DNA synthesizer at a final concentration of .1 M. A separate random bottle mixture of dA, dG, dC and dT phosphoramidites was prepared at respective concentrations such that each base had a 1:1:1:1 coupling efficiency during coupling steps of the 40N random region. In 15 mL of acetonitrile, .363 g of dA, .227 g of dT, .259 g of dG and .387 g of dC were dissolved to make the random mixture bottle 5 g of fresh dicyano imidazole (DCI) was diluted to a final concentration of .25 M in anhydrous acetonitrile and placed on an orbital shaker with molecular sieves for 48 hours prior to DNA synthesis. 7% dichloroacetic acid was prepared in dichloromethane and placed on the DNA synthesizer with fresh bottles of oxidizing solution, Cap A and Cap B mix (Glen Research). The resulting ssDNA library was cleaved from the silica support using concentrated ammonia which was subsequently evaporated off using a cold trap vacuum. The resultant white precipitate was dissolved in Milli-Q H20, pH 7.0. Full length oligonucleotides were gel purified using 8

5.3.2 Biotinylated dsDNA library generation

Gel purified ssDNA library containing a 40N random region was subjected to 2-cycle PCR using a 3 biotinylated primer. The initial melting step was set to 94 C for 2 minutes, the annealing step was set to 55 C for 30 seconds and the extension step was carried out at 71 C for 90 seconds. Each subsequent melting step was carried out at 94C for 30 seconds. The resulting biotinylated dsDNA library was captured on Streptavidin-Agarose beads. The complementary strand was then eluted off of the dsDNA library by adding 200 uL of 20 mM NaOH. The beads were pelleted, the supernatant was removed and beads were washed five times with 1X PBS.

5.3.2 Primer Extension Reaction to Generate Imidazole Modified ssDNA Library

Re-suspended in 1X Primer Extension buffer compatible with Deep Vent (exo-) polymerase. A primer extension reaction was prepared as follows:

Component	Volume	Concentration	
10X Thermopol Buffer	10 uL	1X	
50uM 5' Primer	8 uL	4 uM	
100 mM MgSO4	1 uL	1 mM	
100 mM dACGTP	400 nL	400 uM	
10 mM Imid-mod dUTP	$4 \mathrm{~uL}$	400 uM	
Milli-Q H20	58 uL	_	
DeepVent (exo-) Polymerase	1.5 uL	1.5 U	

All of the reaction components were added to 15 uL streptavidin agarose beads coupled to the ssDNA library template strand, heated to 95 C for 30 s, annealed at 55 C for 5 minutes and incubated at 70 C with orbital shaking at 1000 rpm for 30 minutes. The supernatant was collected and the beads were washed 3X in 1X PBST and the newly synthesized strand containing imidazole modified ssDNA library was eluted from its complementary strand with 20 mM NaOH. The beads were vortexed briefly and spun at 4,500 rpm for 15 seconds. The supernatant containing modified ssDNA library was removed and volume of 80 mM HCl was added to neutralize the pH. The beads were washed 3X in 1X PBST and stored at 4 C with .05 % Sodium Azide. ssDNA was quantified by measuring the absorbance at 260 nm on a spectrohotometer before evaporating the solution to dryness.

Preparation of PEG_3 alkane thiol protected gold nanoparticles

Citrate stabilized gold nanoparticles with a covariation variance of i15 and a mean diameter of 5 nm were treated with 1 mM triethylene glycol mono-11-mercaptoundecyl ether (TEG-MUE) in a 50 ethanol solution in order to create PEG3 alkane thiol stabilizing monolayer around the nanoparticle surface. Gold nanoparticles were placed on an orbital rocker overnight in the 1 mM TEG-MUE ethanol solution. Excess TEG-MUE and ethanol were removed by centrifuging the solution at 13K RPM in order to sediment the nanoparticles at into a visible, deep red concentrated pellet. The supernatant was removed and the nanoparticles were washed 5X with pH 7.0 Milli-Q H20 until there was less than 1 ethanol in solution by volume. The concentration of TEG-MUE protected gold nanoparticles was determined using their absorbance maximum at 520 nm and their cited extinction coefficient at 520 nm. A critical coagulation test was used to visually confirm the stability of the PEG3 alkane thiol protected nanoparticles versus citrate stabilized particles without a capping agent in 1X PBS.

Sequencing of Rd. 8 Pool

TOPO TA vector (Invitrogen) was used to transfect One Shot TOP10 competent E. coli cells to prepare single colonies that were selected based on ampicillin resistance which

is conferred to the cells with the insertion of PCR product in the TOPO TA vector. The general protocol from the manufacturer was followed. 2 uL of fresh PCR product from the Rd. 8 selection step was combined with 1 uL salt solution (1.2 M NaCl, 60 mM MgCl2), 1 uL TOPO TA vector, and 2 uL of Milli-Q H20 to a final volume of 6 uL. 2 uL of the TOPO cloning reaction was added to a vial of One Shot TOP10 compentent E. Coli cells, mixed gently, and incubated on ice for 15 minutes. The cells were heat shocked at 42 C for 30 seconds and immediately transferred to ice. 240 uL of SOC medium was added, the eppendorf tube was capped and shaken at 37 C for 1 hour. Next, a 1:10, 1:50 and 1:100 dilution of the cells were made in 200 uL SOC medium and spread on an LB plate with 100 ug/mL ampicillin with a sterile cell spreader. The cells were incubated overnight at 37 C. The next day, individual colonies were picked and cultured in 2 mL SOC medium overnight in a 5 mL loose screw capped cell culture flask. Plasmid DNA was isolated with a plasmid DNA Miniprep kit (Invitrogen) and sent to Sequeright sequencing service for analysis. The M13 Forward primer was used to identify the integration of DNA into the vector. Sequences were aligned using DOSA (Daughter of Sequence Alignment) software using 80% sequence identity, minimum n-mer of 6, and 80% homology.

Transmission Electron Microscopy Analysis

Samples for TEM analysis were prepared by placing stable solutions of gold nanoparticles formed in the presence or absence of ssDNA on carbon-coated copper TEM grids. The TEM grids were allowed to stand for 2 minutes, excess solution was removed using blotting paper and the grid was allowed to dry prior to measurement. TEM measurements were performed on a Technai F20 instrument operated at an accelerating voltage at 80 kV. Image J was used to calculate gold nanoparticle diameters of the material produced by a randomized pool, an evolved pool and without ssDNA. The set scale tool was used to calibrate the distance in pixels that corresponded to the size of the scale bars. Particle diameters were measured (n = 200) for each sample in order to determine the average nanoparticle diameter and standard deviation. The data were binned in 1 nm increments in order to generate a size distribution plot of the resultant nanoparticle solutions.

Buffers and Cell Culture:

1X PBS - 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM NaH₂PO₄ in Milli-Q H₂0, pH adjusted to 7.4 with 10 M NaOH.

1X SQ20 Buffer (Primer Extension Buffer) - 120 mM Tris-HCl, pH 7.8, 100 mM KCl, 60 mM NH4SO4, 70 mM MgSO4, 1% Triton-X100, 1 mg/mL BSA

1X HotStart PCR Buffer-20 mM Tris-HCl (pH 8.3 at 25C), 20 mM KCl, 5 mM $(NH_4)_2SO_4$

SOC Medium - 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 2% w/v tryptone, 0.5% w/v yeast extract, 20 mM glucose

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