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**14. ABSTRACT**

This project involved investigation and optimization of the chemistry needed to immobilize biorecognition elements (BRE) on gold nanoparticles (AuNPs) to create selective nanoprobes for integration in micro- and nano-fluidic platforms to perform biomarker quantification in biofluids. The AuNPs serve as BRE carriers for analyte pre-concentration to be interfaced with a sensor platform that increases overall sensor sensitivity. Thiol coupling approaches were used in the optimization chemistry to immobilize aptamers and peptides on the AuNP surface. The parameters optimized in this work included reaction times, ligand ratio (PEG-OH vs PEG-COOH) and peptide-AuNP ratio. The first design involving an integrated sensor showed successful cortisol detection at biological levels. A competitive assay involving target recognition at the AuNP surface and release of an electroactive signaling molecule for analyte quantification was realized for cortisol detection. The immobilization protocol was monitored by agarose gel electrophoresis, considering the changes in migration patterns after BRE immobilization as indicators of successful BRE attachment. Peptide coupling was also characterized by dynamic light scattering (DLS) and Zeta potential measurements. The advantage of this approach relies on avoiding surface derivatization on the electrochemical sensor, which can compromise sensitivity and efficiency of signal transduction. Moreover, analyte diffusion to the sensor surface is not required, since the analyte is bound by the AuNPs, which are free in solution, and thus accelerate the detection process.

**15. SUBJECT TERMS**

Biomarkers, Nanofluidics, Pre-concentration Devices, Nanoparticles, Sensing, Human Performance

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**Final Report for AOARD Grant 144087 "Biorecognition element design and characterization for human performance biomarkers sensing"**

**July 16, 2015**

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**Period of Performance:** July 1, 2014-July 1, 2015

**Abstract:** The chemistry needed to perform biorecognition elements (BREs) immobilization on gold nanoparticles (AuNPs) was optimized in order to create selective nanoprobes to be integrated in micro- and nanofluidic platforms to perform biomarker quantification in biofluids.

**Summary:** The expertise of RHXBC researchers in BRE selection, characterization and chemical derivation for sensor development was utilized to creative responsive bio-nanomaterials that recognized biomarkers of interest in Human Performance studies. Protocols with step-by-step instructions for performing peptides and aptamers surface immobilization were provided to collaborators in order to create nanoprobes that were integrated in micro- and nano-fluidic devices. Our first design with the integrated sensor showed successful cortisol detection in biological levels. A competitive assay where target recognition is performed in the surface of AuNPs and an electroactive signaling molecule was released for analyte quantification was realized.

**Introduction:** Selective human performance biomarkers quantification is a challenging task since the typical levels of some of these analytes are orders of magnitude lower than other non-relevant species, like albumins and globulins. Therefore, powerful methods for analyte pre-concentration and/or selective binding in biofluids are needed to create technologies that can monitor biomarker signatures in real-time. The use of nanomaterials as BRE carriers and signal transducing elements for analyte detection has revolutionized the field of diagnostics by providing simple means to design highly selective and rapid biosensors. Antibodies have been the BRE of choice for a few decades now, with great success in the detection of multiple biomarkers related to different conditions. However, their performance in non-ELISA type of formats has been relatively unsuccessful. Moreover, the most sensitive sensing platforms proposed for next generation sensors work based on the transduction of a binding event that occur on the sensor surface. Therefore, the use of BREs that can provide a conformational change upon analyte binding or that due to their small size (< 10 nm) perturb the surface properties after analyte binding is crucial for these sensors' signal generation mechanism. Based on this, small peptides (with sequences made of less than 20 amino acids) and DNA aptamers (via on-off structural switching properties) are appealing BREs for new sensors development. In order to allow their use in different sensor formats the chemistry necessary for successful surface immobilization with preserved activity needs to be optimized.

**Experiment:** The work performed focused on optimizing the chemistry necessary to immobilize aptamers and peptides on the surface of AuNPs. The AuNPs are meant to serve as BRE carriers for analyte pre-concentration to be interfaced with a sensor platform increasing overall sensor sensitivity.
Aptamer immobilization was performed utilizing thiol coupling to the metal surface. To allow the DNA to chemisorb on the AuNPs surface, the DNA was purchased with a 5' end thiol group. Different parameters were optimized for aptamer immobilization, including DNA-AuNP incubation time, salt aging step, and DNA density control through DNA removal with mercaptohexanol (MCH). The optimized protocol is given as an appendix at the end of the document.

Peptide immobilization was performed by immobilizing a PEG ligand containing a carboxylic acid group (-COOH) in one end and a thiol group at the other end. Similarly to the case with the aptamer BREs, the thiol group was used to attach the ligand on the AuNPs surface and the -COOH was used to couple the peptides through their N-terminus. The parameters optimized in this work included reaction times, ligand ratio (PEG-OH vs PEG-COOH) and peptide-AuNP ratio. The optimized protocol is given as an appendix at the end of the document.

In both cases, the immobilization protocol was monitored by agarose gel electrophoresis, considering the changes in migration patterns after BRE immobilization as indicators of successful BRE attachment. Peptide coupling was also characterized by dynamic light scattering (DLS) and Zeta potential measurements.

**Results and Discussion:**

Aptamer immobilization on AuNPs is accomplished by attaching the aptamers through a thiol group in one the ends of the DNA sequence, see Figure 1 for a schematic representation of the process. After the first sequences of DNA have been adsorbed, the high density of negative charges prevents more DNA strands to further reach the surface, therefore, NaCl is added to help reduce the electrostatic repulsion and obtain more densely covered AuNPs. Finally, to reduce surface-aptamer interactions, MCH is used as a filler, to fill the gaps in between the different DNA strands and direct the aptamer sequence to the periphery of the AuNPs, increasing the changes for target binding. Also, the MCH layer would prevent adsorption of non-relevant analytes reducing non-specific binding. The gel in Figure 1 shows the difference in electrophoretic mobilities between AuNPs covered with a non-charge-PEG coating layer and two different AuNPs sets, each coated with a different aptamer. The dramatic difference in mobility is due to the high density of negative charges provided by the DNA strands immobilized on the AuNPs.

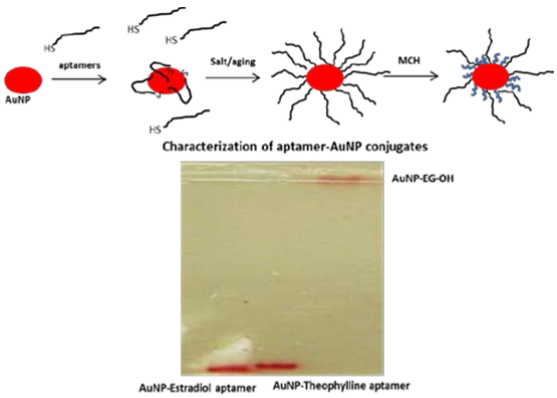


Figure 1. Schematic representation of the DNA immobilization protocol optimized in this work. The gel shows the difference in mobilities between AuNPs coated with a non-charged ligand (PEG-OH) and AuNPs decorated with two different aptamers

A number of small peptides that bind targets related to human performance have been previously selected by AF researchers to expand the capabilities of biomarkers that can be monitored to characterize human performance under different conditions. To immobilize these peptides on metal nanoparticles we decided to use a covalent linkage; contrary to the electrostatically-driven coupling typically performed by some groups. The rationale for this choice is given by the fragility of non-covalent binding of the BRE to the nanoparticle, a covalent bond will allow the use of the BRE-NP conjugates in a range of different conditions, including pH, temperature and salt concentrations, which is critical for the conjugates to be used in biofluids. As shown in Figure 2A, the first step of the ligand immobilization deals with the attachment of the HS-PEG-X molecule. In this step we used a combination of HS-PEG-OH and HS-PEG-COOH in order to control the -COOH density, which later in the process will control the density of peptide immobilized. DLS and Zeta potential measurements confirmed that the ligand mixture was deposited on the AuNPs and that the density of

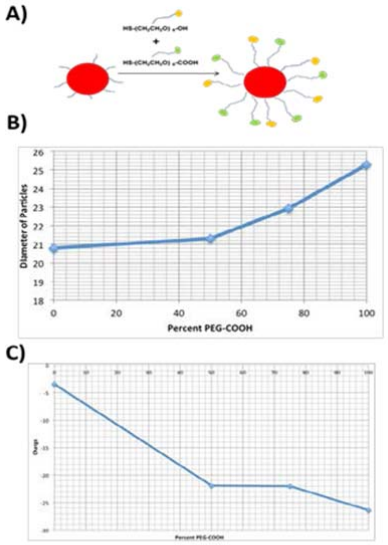


Figure 2. A) Schematic representation of PEG-ligand attachment to AuNPs, B) DLS data showing AuNP diameter changes after different ligand loadings immobilization, C) Zeta pontetial indicating charge changes on the AuNPs surface due to ligand attachment.

–COOH moieties could controlled efficiently.

Peptide attachment was performed using NHS/EDC-catalyzed amine coupling. AuNPs coated with lower densities of -COOH ligands were used, since they provided a wider range over surface charge and density of reactive site. As shown in Figure 3A, the number of peptide units that could be attached to the AuNPs surface is related to the number of -COOH units previously immobilized on the AuNPs. Figure 3B shows the effect of ligand loading on the electrophoretic mobility of the PEG-OH/PEG-COOH coated AuNPs, with significant difference in mobilities between the 10 and 25% -COOH loadings. Therefore these two loadings were further utilized to performance peptide attachment. Figure 3C shows the change in mobility before and after the peptide coupling reaction, showing that in both cases, 10 and 25 % -COOH loading, the AuNPs' mobility was affected by the reaction with the peptide. Importantly, the gel characterization experiments were performed after extending washing to remove peptides that were not chemically attached. Interestingly, peptide immobilization resulted in higher electrophoretic mobilities in the 10% -COOH AuNPs, but resulted in lower mobilities in the 25% -COHH AuNPs. This could be a combination of the effects of the mass and charge added to the AuNPs surface due to the peptide coupling. At lower -COOH densities the charge of the peptide dominated the effect observed, increasing the AuNPs' electrophoretic mobility, while at higher -COOH loadings (probably close to surface saturation) the dominating effect is the added mass of peptide, reducing their mobility. In both cases, peptide immobilization was confirmed by the change in the AuNPs' electrophoretic mobility after the coupling reaction.

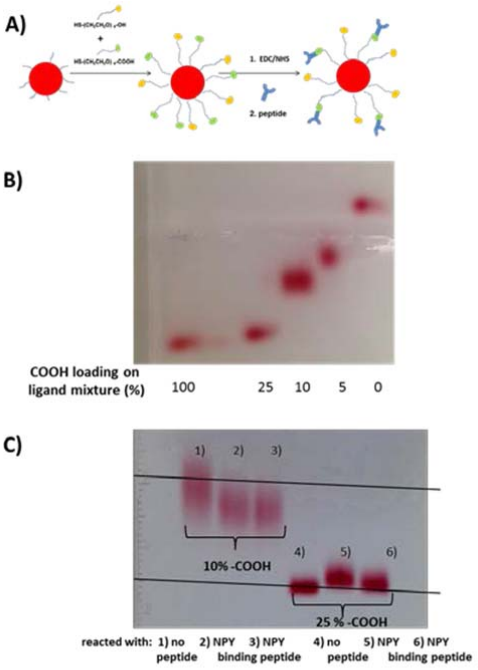


Figure 3. A) Schematic representation of peptide coupling to PEG-AuNPs, B) Agarose gel images comparing the electrophoretic mobility of AuNPs reacted with different PEG-COOH/PEG-OH mixtures. C) Gel images after peptide coupling to AuNP-PEG.

**Integration of aptamer-AuNPs conjugates in sensor platform.**

The aptamer-AuNPs conjugates were integrated in a sensing platform based on a competitive assay that released an electroactive molecule upon cortisol detection. The advantage of this approach relies on avoiding surface derivatization on the electrochemical sensor, which can compromise sensitivity and efficiency of signal transduction. Moreover, analyte diffusion to the sensor surface is not required, since the analyte is bound by the AuNPs, which are free in solution, accelerating the detection process.

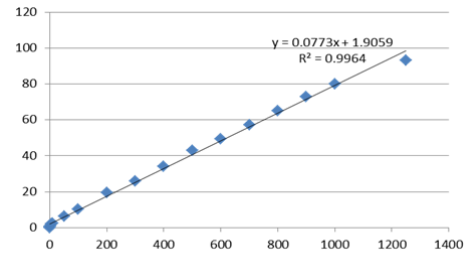


Figure 3. Linear range of sensor response to cortisol.

**List of Publications and Significant Collaborations that resulted from your AOARD supported project:** Peptides were selected through collaboration between AFRL and Wright State University Professor Madhavi Kadakia. The aptamer immobilization protocols developed here were provided to Prof. Nathan Swami to perform the immobilization of a cortisol-binding aptamer on AuNPs. The aptamer was selected by Dr. Jennifer Martin who was a former NRC post-doctoral fellow for Dr. Nancy Kelley-Loughnane at AFRL. The aptamer-AuNPs were utilized to design an assay that detects cortisol using the nano- and micro-fluidics sensor platforms developed in Prof Chia-Fu Chou's (Academia Sinica) and Prof Nathan Swami's (University of Virginia) laboratories.

**Appendix A.**

**Coating AuNPs with thiol-labeled aptamers**

1. Mix 3 mL of 10 nM AuNPs with 45 µL of 200 µM aptamer dissolved in water
2. Allow the mixture to sit in the dark for 45 minutes
3. Add 100 mM PB and 2 M NaCl to obtain a final concentration of 10 mM and 150 mM, respectively:
   Add the 2 M NaCl dropwise and divide the total volume into 5 different aliquots. Add one aliquot every 20 minutes.
4. Let the mixture sit in the dark overnight at RT
5. Centrifuge 200 µL aliquots at 16000 x g for 14 minutes at RT, remove supernatant
   You can centrifuge below RT as well (I have tried this at 18° C), which is helpful because the AuNPs will stick to the bottom of the tube better and it is easier to remove the supernatant.
   You can centrifuge at a lower temperature for any of the AuNPs.
6. Resuspend in 100 µL water, centrifuge at 16000 x g for 10 minutes, remove supernatant
7. Resuspend in 50 µL water, centrifuge at 16000 x g for 10 minutes, remove supernatant
8. Resuspend in 200 µL of 1X PBS pH 7.5
9. Store at 4 °C

M**ercaptohexanol (MCH) treatment of aptamer-AuNPs:**

1. Prepare a 4 µM MCH solution:
   Mix 5.5 µL MCH with 994.5 µL Millipore water (40 mM)
   Dilute by mixing 1 µL of 40 mM MCH with 999 µL water (40 uM)
   Dilute by mixing 100 µL of 40 µM MCH with 900 µL water (4 uM)
2. Mix 28 µL of 4 µM MCH with 1 mL of 10 nM aptamer-AuNPs
3. Vortex for approximately 40 seconds, then allow the mixture to rest for 30 minutes

a. We initially tried only a 5 minute resting time, as the previously optimized procedure indicated. However, we felt that not enough DNA was being kicked off, so we increased the time to 30 minutes and we still ended up with stable AuNPs.

4. Add 1 mL ethyl acetate and vortex for 40 seconds
5. Allow the two phases to separate, then remove the top organic layer with a pipet
6. Repeat this wash two more times
7. Centrifuge 200 µL aliquots at 16000 x g for 14 minutes at RT, remove supernatant
8. Resuspend in 100 µL 1X PBS, centrifuge 16000 x g for 10 minutes, remove supernatant
9. Resuspend in 50 µL 1X PBS, centrifuge 16000 x g for 10 minutes, remove supernatant
10. Resuspend in 200 µL of desired binding buffer, recombine tubes

**Coating AuNPs with peptides using HS-PEG-X ligands aptamers**

**HS-PEG-X Immobilization on AuNPs**

1. Divide 10 mL of AuNP into 20 micro centrifuge tubes (500 µL In each)
2. Prepare 1 mL of 1 mM PEG-OH in millipore $H_2O$

3. Prepare 1mL of 1 mM PEG-COOH in millipore $H_2O$
4. Mix PEG-OH and PEG-COOH at desired levels, e.g. (250 µL PEG-OH and 750 µL PEG-COOH for 75% COOH)
5. Take newly made PEG-OH and PEG-COOH solution and mix with the AuNPs at a ratio of 1 to 25 (20 µL of PEG solution to 500 µL of ~10 nM AuNPs)
6. Place AuNP-PEG tubes in to a vortex and allow to shake vigorously for 30 minutes.
7. Remove Tubes from vortex and place in centrifuge at 14000 RPM for 30 minutes
8. Once centrifuging has occurred for 30 minutes remove tubes and remove the supernatant
9. Replace supernatant with MES Buffer to achieve a final volume of 500 µL
10. Repeat step 8-10 two more times.

**Peptide coupling**

1. Prepare 100 mM sulfo-NHS
2. Prepare 40 mM EDC
3. Pipet 29.5 µL of EDC and 29.5 µL of sulfo-NHS into each micro centrifuge tube containing AuNP-PEG for a final concentration of 5 mM sulfo-NHS and 2 mM EDC
4. Place the tubes into the vortex and allow to shake for 30 minutes to allow the -COOH groups to be activated.
5. While the activation is occurring remove 30 new micro centrifuge tubes and label as desired.
6. After 30 minutes of activation has occurred, split each 500 µL tube into 3x160 µL aliquots in your newly labeled tubes
7. Place all 30 new micro centrifuge tubes into the centrifuge
8. Centrifuge at 14000 RPM for 14 minutes.
9. Remove supernatant and replace with 100 µL of PBS
10. Place tubes back into centrifuge and centrifuge again for 10 minutes
11. Remove supernatant and replace with 100 µL of PBS
12. Centrifuge for 10 minutes
13. Remove supernatant and resuspend to a final volume of 160 µL
14. Ensure peptides are at the same concentration for ease of preparation.
15. Pipet in the peptides so that the final molar ratio of peptides to AuNPs-PEG-COOH is 100 moles of peptide to 1 mole of particles.
16. Place all newly created peptide-AuNP into thermo mixer at 24°C @ 750 RPM for 3 Hours
17. After mixing has occurred remove the tubes and place into centrifuge
18. Centrifuge at 14000 RPM for 15 minutes
19. Remove supernatant and resuspend to a final volume of 160 µL of either PBS or 10 mM HEPES 1 mM $Mg_2Cl$
20. Perform steps 18-19 two more times.
21. Place peptide-coated AuNPs into 4°C refrigerator