POROUS PHOSPHOLIPID NANOSHELL PROTECTED APTAMER SENSOR

FOR URINE MERCURY DETECTION

by

Zhen Li

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APPROVAL BY THEIS DIRECTOR

This Thesis has been approved on the date shown below:

______________________________               ______

11-18-2010

Craig A. Aspinwall                     Date

Professor of Chemistry
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LIST OF ABBREVIATIONS

DOPC: 1,2-dioleoyl-sn-glycero-3-phosphocholine

BisSorbPC: 1,2-Bis[15-(2’,4’-hexadienoyloxy)pentadecanoyl]-sn-glycerol-3-phosphatidylcholine

PPN: Porous phospholipid nanoshell

BSA: Bovine Serum Albumin

Tris: 2-Amino-2-hydroxymethyl-propane-1, 3-diol

SEC: Size Exclusion Column

Quasi Elastic Light Scattering: QELS

Dynamic Light Scattering: DLS

OPA: o-phthaldialdehyde

MPA: 3-mercaptopropionic acid

Hb: Hemoglobin
ABSTRACT

Mercury exposure has been related to neurological diseases and poisoning. Quantification of mercury in biological fluids, such as serum or urine is an important diagnostic method for mercury exposure. We have developed an aptamer-encapsulated porous phospholipid nanoshell (PPN) sensor for sensing mercury in urine using a modified 15-mer single strand DNA. The probe is protected from DNAse and other biofouling species by encapsulation within the porous liposomes composed of mixed phospholipids, allowing direct application of the aptamer in biological fluids containing DNAse and other biofouling materials. The encapsulated sensor was directly tested in urine samples at physiological pH. We were able to detect below 100 ppb (500 nM) Hg²⁺ in urine (urine mercury threshold set by Biologischer Arbeitstoff Toleranz Wert or BAT) with no sample preparation other than pH adjustment. These results suggest that porous phospholipid nanoshells (PPNs) can serve as a general-purpose protection scaffold for biological sensing.
Mercury is toxic in all three forms: elemental, inorganic, and organic (e.g. methyl mercury). Although organic mercury is the most dangerous form, inorganic and elemental mercury are more commonly encountered. Occupational exposure to mercury vapor can lead to high inorganic mercury levels in organs, which has a half-life of years to decades. More recently, the risk of using dental amalgam fillings that contain Hg has been realized and investigated. Currently, the use of traditional Hg dental filling amalgam is banned in Norway, and restricted in Sweden and Finland. Quantitation of urine Hg\textsuperscript{2+} levels is especially important for workers with high occupational mercury (vapor) exposure risks, where routine health evaluation are highly recommended.

Many efforts have been made to monitor Hg\textsuperscript{2+} levels in ecosystems using instrumental method (e.g. ICP-MS), ion-selective membranes/electrodes and small fluorescent indicators. For example, a piezoelectric cantilever method has been used to detect trace Hg vapor and Hg\textsuperscript{2+} in solution. However, none of these methods can directly probe a biological sample such as serum or urine. Ion-selective membranes or electrodes suffer from non-specific interactions with proteins, and since performance depends largely on the surface properties of the membrane or the electrode, such biofouling interferes with measurement of the analytes. The piezoelectric cantilever for unprocessed biological samples is impractical due to the
non-specific interaction with proteins and other species onto the gold film. Small fluorescent molecules have also been synthesized to probe inorganic Hg$^{2+}$ in aqueous solutions; however, they lack sensitivity, are insoluble in water, and require long incubation times with Hg$^{2+}$.\textsuperscript{10}

Aptamer sensors have been applied for detecting mercury in aqueous solutions with excellent selectivity and sensitivity compared to synthetic fluorescent molecules that bind Hg$^{2+}$.\textsuperscript{1,3} However, direct measurement in serum and urine still poses a problem, as nuclease is present in both fluids. However, this limitation can be overcome if the aptamers can be effectively protected from degradation. Recent advances in nanotechnology have provided various macromolecular architectures, such as porous polymer nanoparticles and self-assembled liposomes that can offer protection in biological fluids by providing a physical barrier between the probe and the sample environment.\textsuperscript{11, 12} In this work we demonstrate a porous phospholipid nanoshell (PPN) protected aptamer nanosensor for Hg$^{2+}$.

The sensor was constructed by encapsulating Hg$^{2+}$ sensitive aptamers in a phosphatidylcholine-based nanoshell. Phosphatidylcholine lipids (PC lipids) are the major components of cell membranes and are used as a protective barrier for several reasons: excellent biocompatibility, hollow compartment separated allowing encapsulation, and convenient preparation and purification. The porosity of the nanoshells arises from the use of bis-SorbPC (bis-sorbyl-phosphatidylcholine), which, due to its structure, may form domains of imperfect lipid packing in the
nanoshell architecture. Unlike small organic molecules, the aptamer used in this study has a molar mass of 6 kDa, which is larger than the molecular weight cut off of the porous phospholipid membrane. The size of the aptamer minimizes leakage from the PPNs while allowing sufficient encapsulation efficiency. In comparison, small organic molecules may leak out easily, and larger polymeric materials may suffer from low encapsulation efficiency. The porous and hollow nature of the PPNs provide a protective barrier for the aptamer from DNAse in unprocessed biological fluids, while at the same time allowing small analytes to diffuse into the liposome and interact with the aptamers.

1. 1 Detection Methods for Mercury

A variety of techniques are available for Hg detection, including traditional instrumental methods such as HPLC-ICP-MS or ICP-AES, piezoelectric microcantilevers for Hg vapor detection, ion selective membranes, synthetic Hg binding molecules, surface modified quantum dots and aptamers. Instrumental analysis requires expensive instrumentation and sample preparation. Piezoelectric microcantilevers and ion selective electrodes suffer from biofouling in biological samples. Small fluorophores often have poor solubility in water and low selectivity. Aptamers are more sensitive and selective compared to the small Hg binding fluorophores; however, they cannot be directly used in biological samples due to non-specific interactions with proteins and enzyme digestion. Unlike other
techniques, aptamers can be protected by nanostructures that separate the sensing chemistry from the bulk solution while allowing small analytes to diffuse into the vessels and interact with the aptamer.

1.1.1 Instrumental Methods

Modern advances in instrumentation, including Atomic Absorption Spectroscopy (AAS), Atomic Emission Spectroscopy (AES), and Inductively Coupled Plasma Mass Spectroscopy (ICP-MS), have facilitated the quantification of Hg. High Performance Liquid Chromatography (HPLC) coupled with ICP-MS is a powerful tool to analyze complex samples, and facilitates the measurement of mercury in either inorganic or organic forms. Despite the accuracy and reproducibility generally provided by instrumental techniques, their cost and time-consuming sample preparation usually limit applications where fast and simple analysis is appreciated for diagnostic purposes. Therefore, this thesis will focus on rapid, sensitive assays for Hg$^{2+}$ in urine.

1.1.2 Piezoelectric Microcantilever

Piezoelectric microcantilevers have been used to detection method for elemental Hg. Rogers and coworkers coated a piezoelectric microcantilever with a gold film, which adsorbed mercury vapor and changed the mass and stiffness of the microcantilever, changing the resonance frequency of the piezoelectric material. In
their investigation, Hg vapor in nitrogen was detected as low as 93 ppb was detected.\(^8\)

Xu and coworkers extended this method to detect Hg\(^{2+}\) in aqueous solutions.\(^{22}\) They were able to show that the rate of cantilever bending depended on the concentration of Hg\(^{2+}\) ions. Other ions such as: Na\(^+\), K\(^+\), Pb\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\), Cu\(^{2+}\), Ni\(^{2+}\) and Ca\(^{2+}\) were used to test the selectivity of the sensor, but none of these ions showed the same level of cantilever deflection as Hg\(^{2+}\). Hg\(^{2+}\) as low as 10\(^{-11}\) M could be detected.

1.1.3 Ion Selective Dyes and Membranes

Polymeric ion selective membranes have been prepared to detect Hg\(^{2+}\) in aqueous solutions. Two different detection mechanisms are typically employed: fluorescence and potentiometry. Hassan and coworkers developed a polyvinyl chloride (PVC) based ion selective membrane by doping the polymer with ethyl-2-benzoyl-2-phenylcarbamoyl acetate (EBPCA), which can selectively bind Hg\(^{2+}\) in aqueous solution.\(^{14}\) The structure of EBPCA is shown in Figure 1.1. A transparent and homogenous polymer membrane was obtained (0.2 mm thickness and 11 mm in diameter) and used for electrochemical measurements. Although the membrane achieved high selectivity against Co\(^{2+}\), Cu\(^{2+}\), Ni\(^{2+}\), Cd\(^{2+}\), Fe\(^{3+}\), Pb\(^{2+}\) and La\(^{3+}\), the detection limit was 10\(^{-6}\) mol/L, higher than the mercury threshold in biological samples.\(^1\) Biofouling of the electrode surface may also interfere with
measurement in biological samples, thus efficient sample preparation is required to remove the proteins and other large molecules to “clean” the solution.

![Molecular structure of EBPCA for potentiometric measurement of Hg²⁺.](image)

Figure 1.1.1 Molecular structure of EBPCA for potentiometric measurement of Hg²⁺.

Chan and coworkers designed an fluorescence based ion selective membrane with 5,10,15,20- tetraphenylporphorin (H2tpp) dissolved in PVC.¹⁸ Hg²⁺ was extracted into the hydrophobic membrane and where it formed metalloporphyrin complexes (Figure 1.2), leading to fluorescence quenching of H2tpp. Selectivity against Cd²⁺, Cu²⁺, Fe³⁺, Ag⁺ and Pb²⁺ was high, and a detection limit of 40 nM at pH 8.0 was obtained. Although their membrane showed promising results in simple aqueous samples, measurements in biological fluids were not attempted.
Small fluorescent indicators whose optical properties are sensitive to Hg$^{2+}$ can also be used for detection. Rurack and coworkers developed a small fluorescent sensor (compound A) for heavy transition metals (Figure 1.3).\textsuperscript{19}

Once bound to either Hg$^{2+}$, Cu$^{2+}$ or Ag$^+$, the quantum efficiency of compound A is enhanced. Although only two other ions cause a fluorescence change when bound to compound A, poor solubility in aqueous media limit the use of compound A to organic solvents such as acetonitrile. Other small fluorescent molecules have
also been synthesized to detect Hg$^{2+}$; however, they generally suffer from low selectivity and poor solubility in water.\textsuperscript{23}

1.1.4 Quantum Dots

Semiconductor nanoparticles (quantum dots, QDs) that are highly fluorescent (CdS, CdSe, ZnSe, etc.), have drawn considerable attention due to their fascinating optical properties. CdSe, for example, can be tuned to fluoresce across the entire visible spectrum by changing the particle size due to quantum confinement.\textsuperscript{24} QDs have very promising optical properties, but they cannot be used for sensors without modification, as they do not inherently have the ability to transduce an event into a signal. Xiang and coworkers used mercaptopropionic acid capped CdSe quantum dots to detect Hg$^{2+}$, Ag$^{+}$, and Cu$^{2+}$.\textsuperscript{20} When the modified QDs were mixed with the metal ions the fluorescence intensity was quenched. Although the detection limit for Hg$^{2+}$ was found to be 3 nM, the exact mechanism for the quenching phenomenon is still unclear. The mercury response curve can be found in Figure 1.4.
Figure 1.1.4. Mercury response from mercaptopropionic acid modified CdTe quantum dots. The linear range is from 0.003 to 2.4 µM. HAP, hydroxyapatite, was used to enhance the sensitivity of the quantum dots to Ag⁺ and Cu²⁺, although no enhancement was observed for Hg²⁺. Reproduced from reference #58.

QDs are more photostable, and have a tunable emission profile, a higher extinction coefficient and a longer fluorescent lifetime compared to organic dyes. Furthermore, quantum dots with different sizes can be simultaneously excited which makes them ideal for labeling and multiplex assay. However, quantum dots themselves are inadequate for sensors, due to the lack of transducing properties. A layer of surface ligands is needed, and the surface must be passivated to prevent non-specific interactions in complicated systems such as biological fluids.

1.1.5 Aptamer

Aptamers are oligonucleic acids (DNA or RNA) or peptides that can bind specific analytes. DNA aptamers have a greater stability compared to RNA aptamers due to structural differences. Aptamers are selected for specific analytes.
by “Systematic Evolution of Ligands by Exponential Enrichment” or SELEX.\textsuperscript{27,28} Generally, a large pool of random DNA is chosen and loaded onto an affinity column with stationary phase modified by desired substrates. DNAs that do not bind to the targets are removed, while DNAs that show a strong affinity are collected and amplified by PCR for the next round of selection. After several cycles, selective and sensitive binding sequences can be obtained. The advantages of aptamers over antibodies are obvious. Theoretically, aptamers can be selected for any analyte by SELEX, and modified aptamers can be chemically synthesized rather than harvested from cell lines and then purified, as required for antibodies.

Modified aptamers have been used to examine Hg\textsuperscript{2+} in aqueous solutions.\textsuperscript{10,21} Ono, et.al found that thymine-thymine pairs bind Hg\textsuperscript{2+} ions in DNA duplexes,\textsuperscript{10} and thus developed an oligodeoxyribonucleotide (ODN) based sensing system to detect mercury. Upon Hg\textsuperscript{2+} binding, the aptamer structure “folds” so that a fluorophore at one end and a dabcyl at the opposite end of the aptamer are in close proximity, quenching the fluorescence of the fluorophore (Figure 1.5). This aptamer sensor is more sensitive than existing small fluorescent molecules and more selective over other heavy metal ions.\textsuperscript{10}
Chang and coworkers also used aptamer-based sensors to detect Hg$^{2+}$ in environmental samples using a 15-mer single strand DNA.\(^{21}\) The two ends of the aptamer were modified by attaching a fluorophore (6-FAM) and a quencher (dabcyl). This particular sequence can be used to detect Hg$^{2+}$ in the range of 50~1000 nM or 1~10 mM for K$^+$ (Figure 1.6). Both aptamer sensing systems showed promising selectivity and sensitivity compared to small fluorescent molecules. They are both structurally stable and soluble in aqueous solutions. However, they cannot be directly used in biological fluids such as serum or urine, since DNAse could easily digest and degrade the DNA-based aptamer.
1.6 Sequence of the aptamer and the sensing scheme of 15 mer single strand DNA aptamer.

1.2 Nanovessels

It is advantageous to incorporate a protection mechanism to preserve the activity of sensing chemistry in complicated biological samples, as non-specific protein interactions and enzyme digestion may skew quantitative analyte measurement. Sequestering the sensing chemistry from the bulk sample and limiting the diffusion of biofouling agents would improve the sensitivity and selectivity of the sensor. Probes Encapsulated By Biologically Localized Embedding (PEBBLEs) or hollow porous phospholipid nanoshells (PPNs) are nanostructures that can serve as protective carriers. The porous nature of the PEBBLEs allows only small molecules to diffuse into the cavities of the nanoparticle, while keeping larger
species (such as proteins) outside. PPNs, on the other hand, are self-assembled structures that consist of porous lipid bilayers that separate the inner compartment from the bulk solution. The porous lipid bilayer serves as a dialysis membrane that allows only small analytes to migrate into the inner compartment but prevents the entrance of larger species. Both approaches have their own unique advantages and disadvantages.

1.2.1 PEBBLES

Probes Encapsulated By Biologically Localized Embedding (PEBBLEs) are polymeric sub-micron probes designed for real-time intracellular sensing and imaging with minimal impact on the cellular environment by protecting the fluorophore within from biofouling and non-specific protein interactions. Originally synthesized and investigated by Kopelman and coworkers, PEBBLEs are among of the first intracellular nanosensors. PEBBLEs are usually constructed from one of two polymers: polyacrylamide or poly (decyl methacrylate). Polyacrylamide PEBBLEs have been synthesized for pH, oxygen and calcium sensing using a water-in-oil emulsion method. For encapsulation, fluorescent dyes are mixed with monomer during the synthesis, which results in a polyacrylamide nanobead with fluorescent dyes trapped inside the polymer network. Typically, two fluorescent dyes are used; an indicator dye and a reference dye, enabling ratiometric sensing.
Leakage, due to the porous nature of the polymer, limits the practical use of many dyes within PEBBLEs, although leakage is minimized when short measurement windows are utilized. PEBBLEs are designed for single use and due to the time scale of single cell measurement, some leakage is often acceptable, particularly when an internal reference dye is used. Due to the small size of the PEBBLEs, hundreds or thousands can be introduced into the intracellular environment without occupying a substantial volume fraction. Because most of the sensing chemistry is inside the polymer network, analytes must diffuse into the PEBBLEs to generate a signal. Therefore the pore size of the PEBBLEs intrinsically limits the size of the analytes that can be investigated, since only relatively low molecular weight analytes can diffuse into the polymer. Analytes such as H\(^+\), Ca\(^{2+}\), Mg\(^{2+}\), Cu\(^{2+}\), Cu\(^+\), Cl\(^-\), Zn\(^{2+}\), O\(_2\), glucose, and H\(_2\)O\(_2\) have been studied using PEBBLEs.

PEBBLEs provide excellent protection from biofouling. When free fluorescent dyes, such as carboxynaphthofluorescein, were mixed with albumin solution (0.01%), a 90% increase in fluorescence intensity was observed. This abnormal emission makes the direct measurement using free fluorophores unpredictable and unreliable. However, when the same dyes were encapsulated in PEBBLEs, only a negligible increase in fluorescence intensity was observed because of the blocking effect provide by the PEBBLEs.

In addition to sensors based on the encapsulation of small dyes, a glucose
sensitive PEBBLE was prepared by trapping glucose oxidase within polyacrylamide. Oregon Green 488-dextran or Texas Red-dextran was used as a reference dye while Ru([dpp(SO₃Na)₂]₃)Cl₂ was co-encapsulated as the reporter dye. Glucose oxidase catalyzed the oxidation of glucose into D-glucono-δ-lactone, depleting dissolved oxygen, lessening the dynamic quenching of Ru([dpp(SO₃Na)₂]₃)Cl₂ and resulting in an increase of fluorescence intensity. The sensor also demonstrated resistance to biofouling from bovine serum albumin (BSA) compared to free Ru([dpp(SO₃Na)₂]₃)Cl₂, although the response time for these sensors was insufficient for dynamic intracellular measurement.

Recently, Ozalp and coworkers demonstrated the possibility of encapsulating aptamers inside polyacrylamide nanoparticles for intracellular ATP sensing. The structure of the aptamer is show in Figure 1.7. Upon ATP binding, the conformation change of the DNA separates the fluorophore/quencher pair, resulting in an increase of fluorescence intensity. However, direct use of aptamer-based probes in vivo could be problematic due to the presence of endo- or exo- nucleases as well as DNA binding proteins as shown by Wang and coworkers.
Although aptamers have well defined chemistry, the incorporated fluorescent labels suffer from the same interference from proteins as small dye molecules. In addition, nucleases can degrade the nucleotide-based aptamers. Once encapsulated within polyacrylamide PEBBLEs, protected aptamers showed excellent response to spiked ATP in the presence of DNase, while free aptamers showed no response to ATP after 2 hours of digestion in DNase solution. Thus, PEBBLEs protect fluorophores from biofouling and aptamers from enzyme digestion. However, radicals generated during the encapsulation and polymerization process may affect the activities of encapsulated aptamers. It is known that free radicals can cause indirect DNA damage in mitochondria, and in the research of Ozalp et al., a significant decrease in signal after polymerization and encapsulation was observed. This decrease was partially due to the radical damage.

1.2.2 Phospholipid Nanoshells
1.2.2.1 Phospholipids

Since the first observation of phospholipid liposomes by Bangham in 1965, lipid-related research has drawn considerable attention. Phospholipid liposomes are super molecular structures that mimic cell membranes. Liposome architecture consists of a spherical lipid bilayer separating a small inner volume from the bulk solution. Sensing chemistry can be encapsulated in the interior volume so that undesired interactions with complex samples can be minimized by the lipid bilayer.

Phospholipids, the basic building block for liposomes, are a class of amphiphilic molecules, with a hydrophilic head group and a hydrophobic tail, similar to common surfactants such as sodium dodecyl sulfate (SDS). However, unlike common surfactants that have a critical micelle concentration (CMC) in the range of $10^{-2}$-$10^{-4}$ M, bilayer-forming lipids have a CMC that is 4 to 5 orders of magnitude lower because they have much lower solubility in water.

Diacylglycerides phospholipids are the most studied because they can be harvested from biological samples and are the major components of cell membranes. In a phospholipid molecule, two out of the three hydroxyl groups in the glycerol backbone form ester bonds with fatty acids, constituting the hydrophobic tail region of the lipid molecule. The phosphate group forms another ester bond with the last hydroxyl group to form the hydrophilic head group. The head group can be modified by other functional groups to form more complex hydrophilic structures. Common head groups are listed in Table 1.1.
Table 1.1 List of phospholipid head groups.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Name</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Phosphatidylethanolamine" /></td>
<td>Phosphatidylethanolamine</td>
<td>PE</td>
</tr>
<tr>
<td><img src="image" alt="Phosphatidylcholine" /></td>
<td>Phosphatidylcholine</td>
<td>PC</td>
</tr>
<tr>
<td><img src="image" alt="Phosphatidylglycerol" /></td>
<td>Phosphatidylglycerol</td>
<td>PG</td>
</tr>
<tr>
<td><img src="image" alt="Phosphatidylserine" /></td>
<td>Phosphatidylserine</td>
<td>PS</td>
</tr>
</tbody>
</table>

Among the different head groups in Table 1.1, phosphatidylcholine lipids are the primary component of biological membranes. PC lipids are easily obtained from various sources such as soybeans and egg yolk. As with the head groups, the hydrophobic tails of the lipid can vary. Two fatty acids form ester bonds with hydroxyl groups from the triglycerol backbone. The lipids can have either the same or different fatty acid chains. Common lipid tail structures are shown in Table 1.2. Structures of the phospholipids used in our work are shown in Figure 1.8.
Table 1.2.1 Fatty acid structure and nomenclature.

<table>
<thead>
<tr>
<th>Fatty Acid Structures</th>
<th>Abbreviation</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14:0</td>
<td>Myristic Acid</td>
</tr>
<tr>
<td></td>
<td>16:0</td>
<td>Palmitic Acid</td>
</tr>
<tr>
<td></td>
<td>18:0</td>
<td>Stearic Acid</td>
</tr>
<tr>
<td></td>
<td>18:1(^9)</td>
<td>Oleic Acid</td>
</tr>
<tr>
<td></td>
<td>18:1(^9t)</td>
<td>Elaidic Acid</td>
</tr>
<tr>
<td></td>
<td>16:2(^2t,4t)</td>
<td>Sorbyp-substitute</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ddecanoic Acid</td>
</tr>
</tbody>
</table>

Notes:

- **18:1\(^9\)**: Position and Isomerism of the unsaturated bonds
- Carbon Chain Length
- # of unsaturated bonds
Bis-SorbPC Figure 1.2.3 (B) has ester bonds in the hydrophobic tail region of the fatty acid moiety. This unique structure may affect the packing of bis-SorbPC in the bilayer thus introducing porosity on the lipid membrane. To prove the porous nature of the bis-SorbPC bilayer, Cheng and coworkers used fluorogenic reactions based on primary amine coupling with OPA (o-phthaldialdehyde) in the presence of 3-mercaptopropionic acid (MPA) (reaction shown in Figure 1.2.4). Hemoglobin was used to provide accessible primary amines and encapsulated inside the
unpolymerized bis-SorbPC vesicles. The fluorescence emission only occurred when all 3 compounds are present. Therefore, by introducing OPA and MPA outside the liposomes, it is possible to evaluate the permeability of the bis-SorbPC membrane.

Figure 1.2.2 Fluorogenic reaction (A) and normalized fluorescence spectra from primary amine labeling reaction (B). In plot B, spectra are obtained for (a) OPA-MPA; (b) Hb encapsulating DOPC with OPA-MPA; (c) Hb encapsulating bis-SorbPC with OPA-MPA; (d) Hb encapsulating polymerized bis-SorbPC/EGDMA with OPA-MPA; and (e) the same as (b) and (c), but with addition of excess Triton X-100 to disrupt vesicles.

Shown in Figure 1.9, in contrast to 14% increase in normalized fluorescence intensity from DOPC liposomes, unpolymerized bis-SorbPC vesicles yielded 64% increase in fluorescence intensity indicating more readily migration of MPA and OPA through the lipid bilayer namely the porous nature of the membrane. After bis-SorbPC was polymerized with EGDMA, the porous nature of the liposomes was retained.
It should be noted that membrane porosity is a key requirement to construct encapsulated sensors. The porous membrane will only allow small molecules to diffuse in or out while blocking the passage of large molecules. When probing biological samples, non-specific interaction with macrobiological molecules such as proteins is usually undesired and thus must be minimized. The unique property of the bis-SorbPC vesicles can provide excellent protection from interaction between bio-fouling agent and sensing chemistry, while facilitating analytes access.

1.2.2.2 Liposomes

The amphiphilic property of the phospholipids facilitates the formation of self-assembled macrostructures in an aqueous environment by minimizing the unfavorable interactions between the non-polar fatty acid chains and the bulk solvent. Several possible supramolecular structures exist including micelles, multilamellar or unilamellar liposomes, a hexagonal phase, etc. In addition, the self-assembled structure depends on the molecular shape of the amphiphiles. We can define the hydrophobic region of supramolecular structure as $S_{np}$, and hydrophilic region $S_p$. The relative sizes of the two regions determine the morphology of the supramolecular structure. For example, if $S_p$ is larger than $S_{np}$, micelle formation is favorable; if the two areas are comparable, a bilayer structure is favorable. The relationship between the shape and size of the amphiphiles and the super molecular structure is illustrated in Figure 1.10
Figure 1.2.3. The impact of the morphology of amphiphiles on the supramolecular structure. Reconstructed from reference #27

With low spontaneous curvatures for the PC and PS phospholipids, bilayer structures are favored. However, curvature depends on the relative amount of water in a lipid/water mixture. The non-polar tails tend to expand with decreasing water content, which favors the inverse hexagonal phase (Figure 1.11). Temperature and addition of cholesterol will also affect curvature, where increases in both increase the curvature, favoring the inverse hexagonal phase.
Within the lamellar phase (bilayer) exists temperature-dependent sub-phase transitions. The sub-phase can be divided into the crystalline (solid-like), the ordered (gel-like), and the disordered (liquid, liquid-crystalline) membrane phases according to the order of the hydrophobic chains. The transition between the three phases is depicted in Figure 1.12.
The transition temperature $T_m$ increases as carbon chain length in the fatty acid moiety increases for saturated phospholipids due to increasing Van der Waals forces. Partitioning cholesterol into the bilayer also increases the transition temperature. Incorporating unsaturated groups into the alkyl chains decreases the transition temperature, due to the fact that double or triple carbon-carbon bonds will prevent lipids from packing as closely as fully saturated hydrocarbon chain. This disruption increases the fluidity of the bilayer and decreases the transition temperature. At $T_m$, the two phases of the lipids coexist in the bilayer and form different domains, causing more structural defects and leading to greater leakage of small molecules through the bilayer. This increased permeability of the bilayer can be used as a release mechanism for entrapped cargo. Depending on the bilayer composition, release of the cargo can be controlled. For sensing purposes, the porous structure can serve as a molecular weight barrier similar to a dialysis membrane. By
adjusting bilayer composition, a membrane through which small analytes can easily diffuse but large species cannot either enter or exit can be fabricated.

Several phospholipid liposome preparation strategies have been developed to yield different types of liposomes. Multilamellar liposomes (MLV) are closed concentric lipid bilayers separated by aqueous channels. Unilamellar liposomes (ULV) are single shell spheres with one bilayer separating the interior aqueous compartment from the bulk solution. The ULV can be further divided into several categories based on size. Small unilamellar liposomes (SUVs) range from 50 to 100 nm in diameter, large unilamellar liposomes (LUVs) are typically 100 to 500 nm in diameter, and giant unilamellar liposomes (GUVs), have diameters larger than 2 μm. An illustration of MLV and ULV is shown in Figure 1.13.
Liposomes can be prepared by hydrating dry lipids with either water or a buffer solution, yielding MLVs. Although multi-concentric bilayers in MLVs are suitable for X-ray studies, their heterogeneity and complex interior compartments make them unfavorable candidates for bilayer property studies such as permeability. ULVs can be prepared from MLVs by extrusion,\textsuperscript{51} sonication,\textsuperscript{52} or by modifying the hydration procedure so that the formation of unilamellar liposomes is spontaneous.

Figure 1.2.6 Classification of phospholipid liposome structures based on lamellarity and sizes
LUVs are more stable than the SUVs due to their larger radius of curvature. LUVs also have larger encapsulation volume, making them better candidates for delivery or encapsulation purposes.

The extrusion method is usually employed to control the size of the liposomes. Phospholipids are typically stored in a hydrophobic and volatile solvent, such as chloroform. The lipids are first dried, so that a semi-transparent phospholipid thin film is obtained on the wall of the container. After 4 hours of further drying under reduced pressure, the film is then hydrated with a buffer solution. MLVs are usually obtained after hydration. Freeze-thaw cycles can then be used to homogenize the MLVs. The MLVs are then extruded through porous polymer membranes to prepare uniform liposomes. This method is limited to the fabrication of LUVs; sonication is generally sufficient for the formation of SUVs.

ULVs can also be prepared via solvent injection, detergent dialysis, and electroformation. During solvent injection procedures, phospholipids are typically dissolved in ethanol, and then slowly injected into an aqueous solution. The ethanol is considered instantaneously diluted in the bulk solvent, and the phospholipids eventually form liposomes. This is a simple, mild and homogenous preparation. Unlike the film hydration method, this technique can be used to encapsulate some lipophilic species that are soluble in ethanol. However, the liposome concentration is limited by the solubility of the phospholipid in ethanol, the concentration of the prepared liposomes is further limited by the maximum amount of the ethanol that
could be injected to aqueous solution (7.5% by volume), and the encapsulation efficiency is also low if the species intended to be trapped readily dissolves in aqueous solution.\textsuperscript{55}

Detergent dialysis was first described by Rhoden and coworkers.\textsuperscript{56} Phosphatidylcholine and cholesterol solutions are mixed, dried under an inert gas (N\textsubscript{2}), and then dried further under reduced pressure. The mixture is hydrated in a buffer solution containing cholic acid. A small aliquot of the mixed solution is then immersed in a boiling water bath for 30 seconds and cooled on ice immediately. The liposomes are formed by the removal of the detergent by dialysis for 16 hours. This approach yields mostly unilamellar and uniform liposomes where the dimensions of the liposomes can be tuned by changing the ratio between the phospholipids and cholesterol, as well as by adjusting the pH of the dialysate.\textsuperscript{56}

Electroformation is widely used to prepare GUVs. Angelova and coworkers\textsuperscript{57} deposited a mixture of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine dissolved in a 9:1 ratio of chloroform and methanol on a platinum electrode. The volatile solvent was evaporated under nitrogen gas and replaced by an aqueous solution. A dc voltage (<3 V) was applied to the electrode. The rate of liposome formation depends on the temperature: liposomes form in 24-48 hours at 4\textdegree C or 30 minutes at 70\textdegree C. A possible limitation of this technique is that liposomes will not develop under high ionic strength (when the concentration of monovalent salt is above 10\textsuperscript{-4} mol/L).\textsuperscript{57}
In this work, we utilized the extrusion method to make homogenous, unilamellar liposomes with pre-defined diameters. This process is illustrated in Figure 1.14.

![Figure 1.2.7 Simplified phospholipid liposome preparation scheme.](image)

1.2.3 Conclusion

The two common nanosensor platforms discussed here have unique advantages and disadvantages. PEBBLEs show resistance against biofouling, low leakage of fluorescent dyes, and promising results with intracellular sensing. However, the radical initiators employed during the polymerization process can damage the entrapped molecules. Liposomes are analogous to cell membranes, providing improved biocompatibility for intracellular applications. The preparation
of phospholipid liposomes and encapsulation inside the nanostructures is a simple physical process, and since the porosity can be controlled by adjusting lipid composition, they are excellent candidates for sensor platforms that require restricted access to entrapped probes.

1.3 Development of Porous Phospholipid Liposome Protected Aptamer Sensor

Aptamers have two advantages over other sensing techniques: higher solubility in water and greater sensitivity and selectivity toward Hg$^{2+}$ compared to small Hg$^{2+}$ sensitive fluorophores. In order to exploit these properties in biological samples, a protective mechanism must be employed. PEBBLEs and liposomes both show resistance against biofouling and a capacity for encapsulation. Due to the ease of fabrication and purification of the liposomes without exposing the aptamer to potentially damaging radicals or detergents, we used porous phospholipid nanoshells to examine the Hg$^{2+}$ levels in biological fluids.

In this thesis, we demonstrate a sensor system employing Hg$^{2+}$ binding aptamers encapsulated in porous phospholipid nanoshells for direct detection of Hg$^{2+}$ in urine (Figure 1.15). The aptamer we used is a 15-mer modified single strand DNA aptamer previously studied by Chang et al. The multiple thymine pairs bind Hg$^{2+}$ and fold the DNA so that the fluorophore and quencher are in close proximity, causing fluorescence quenching. To make PPNs, we used a 1:1 mixture of DOPC and BisSorbPC. The two sorbyl groups in the tail regions of the BisSorbPC alter the
packing pattern in a bilayer compared to the saturated PC lipids, resulting in structural defects and porosity of the membrane. This membrane porosity is essential for sensing because it provides a passage for the analytes to access the encapsulated sensing chemistry. To illustrate the importance of porosity, we used 100% DOPC liposomes to encapsulate the aptamers as a control study.

As shown in Figure 1.15, the two lipids are mixed and tried then hydrated with aptamer solution. After freeze-thaw, extrusion and purification, PPNs with encapsulated aptamers were prepared. The pores on the PPNs allow analytes such as Hg$^{2+}$ to freely diffuse in or out of the vesicles, and thus interact with the sensing chemistry. Several key objectives were investigated and will be discussed in the following chapters, including protection from BSA biofouling and DNAse digestion, and detection of Hg$^{2+}$ in human urine without any sample purification.

Figure 1.3.1 Illustration of Hg$^{2+}$-sensitive PPN sensor developed in this work.
Using this PPN sensor system, we were able to detect as low as 50 nM Hg$^{2+}$ in urine without any purification of the sample, reaching the detection threshold for Hg$^{2+}$ in urine set by WHO.$^1$ We believe PPNs can serve as a general encapsulation platform for other sensor and delivery applications.
CHAPTER 2: MATERIALS AND METHODS

2.1 Materials and Solutions

Mercury acetate was purchased from Strem Chemicals, (MA, USA); Aptamer (5’-FAM-GGT-TGG-TGT-GGT-DABCYL-3’) was purchased from Integrated DNA Technologies Inc. (Coralville, IA); DNase I (from bovine), Tris(hydroxymethyl)-aminomethane (Tris) and Bovine Serum Albumin (BSA) were purchased from Sigma-Aldrich. Anhydrous sodium acetate was obtained from Mallinckrodt AR, (KY, USA). KCl was obtained from EMD Chemicals. (NJ, USA). 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids(AL,USA). 1,2-Bis[15-(2’′,4’′-hexadienoyloxy)pentadecanoyl]-sn-glycerol-3-phosphatidylcholine (Bis-Sorb PC) was synthesized and purified as previously described.\textsuperscript{58} Human urine was collected from a healthy adult.

Tris acetate buffer (10 mM pH 7.4) was filtered through a polycarbonate membrane with 200 nm pore size and autoclaved for 40 minutes. An aptamer stock solution was made by dissolving in Tris acetate buffer with a final aptamer concentration of 100 µM. A stock KCl solution was made by dissolving in nanopure water with a concentration of 0.4 M. Mercury acetate was initially dissolved in 0.1 M HCl to make a 2.5 mM solution, and then diluted with nanopure water to make 5 and 50 µM stock solutions. No precipitates were observed.
2.2 Instrumentation and Apparatus

Fluorescence measurements were performed on a Photon Technology International (PTI) QuantaMaster 40. The excitation wavelength was set at 490 nm. All slits widths were set at 4 nm/1.0 mm. BSA solution and urine backgrounds were collected and subtracted from the raw spectra. Scattering backgrounds of the vesicles were obtained as follows: first measure the fluorescence spectra of aptamer encapsulated vesicles was measured before and after vesicles lysis, and the difference between the two spectra was used for scattering background subtraction.

The dynamic light scattering (DLS) experiments were conducted on a BI8000 autocorrelator from Brookhaven Instrument Corp. The scattering angle was fixed at 90°, and the hydrodynamic diameter was calculated based on non-negatively constrained least squares fitting. Experiments were performed at room temperature (25°C) in a refractive index matching solution.

2.3 Preparation and Purification of Vesicles

Porous, unilamellar phospholipid vesicles were prepared by the film hydration technique. A 1:1 mass ratio (total weight: 2 mg) of DOPC and Bis-Sorb PC in chloroform was mixed and dried under nitrogen. The mixture was further dried under vacuum overnight to remove residual solvent. For encapsulation, 25 μL of the aptamer stock solution was first mixed with 175 μL Tris-acetate buffer ([aptamer] = 12.5 μM), and then added to the dry lipids. After 11 freeze-thaw cycles
(solution was alternatively placed in dry-ice/isopropanol (−78°C) and a 42°C water bath), the lipid solution was extruded 21 times through Whatman polycarbonate membranes with an average pore size of 200 nm using a mini-extruder from Avanti Polar Lipids (Birmingham, AL). The extruder was slightly heated (40-50°C) to facilitate the extrusion process for 1:1 lipid mixtures. (For DOPC, the extruder was used without heating.) The vesicle solution was then collected and purified by size-exclusion chromatography (SEC). Sepharose CL-4B (Sigma-Aldrich) was packed in a 0.7×20 cm Flex Column from Kontes (USA). The column was conditioned with the degassed Tris-Acetate buffer for 2 hours before use. A total of 8 fractions were collected at 2 mins (~0.4-0.5 mL per fraction) increment. Fraction No. 6 was confirmed to contain vesicles by DLS, where a size distribution centered at 100-120 nm was observed. DOPC vesicles were prepared following the same procedures using 25 μM aptamer solution for hydration.

* Note: In our first investigation, we used 100 μL of 100 μM aptamer solution mixed with another 100 μL buffer to hydrate the 1:1 mixed lipids. This batch of vesicles was used to obtain the mercury calibration curves. The solution used for calibration study was 15 μL of the purified vesicle solution with 785 μL buffer solution. For DOPC vesicle preparation, 50 μL of 100 μM aptamer was mixed with 150 μL buffers. We used smaller volumes of the 100 μM aptamer stock solution as our research progressed in order to conserve the limited amount of aptamer.
2.4 Calibration Curves

In order to generate calibration curves for our sensors, a 12.5 nM solution of aptamer in Tris-acetate buffer was spiked with 40 or 400 mM stock K\(^+\) solution to give final K\(^+\) concentrations of 0.10, 0.49, 0.98, 2.9, 4.8, 6.7 and 9.6 mM. For testing mercury response, small volumes of Hg\(^{2+}\) stock solutions were spiked into 12.5 nM aptamer solutions to yield final Hg\(^{2+}\) concentrations of 50, 98, 192, 370, 535 and 833 nM. The final concentrations and fluorescence intensity were corrected for the effect of dilution. All experiments were performed in triplicate, and for all aptamer response studies, spiked solutions were always incubated for 10 minutes before fluorescence intensity was measured. In addition, a time-based study was also performed in order to determine the incubation time. The fluorescence of the mixed solution was measured every 5 minutes from 5 to 15 minutes. No change of intensity was observed after 5 minutes of mixing.

2.5 DNAse Digestion and BSA Bio-fouling Test

Solutions of 3mM Mg\(^{2+}\) and Ca\(^{2+}\), 1 μM of free aptamer or 5 μL encapsulated aptamers and 5 μL DNAse were combined, and incubated at 37 °C for 1 hour. The sample was diluted to 400 μL with Tris-acetate buffer prior to fluorescence measurement. Stock KCl solution was spiked into the mixture to give final K\(^+\) concentration of 9.76 mM.

To demonstrate the protection offered by the PPNs for encapsulated
aptamers, small fractions of a stock BSA solution were spiked into an aliquot of encapsulated aptamer solution (375 μL buffer with 5 μL encapsulated aptamer solution) to make 0.1%, 0.3% and 0.5% BSA concentrations. For comparison, 5 μL free aptamer solutions were mixed with 380, 350 and 315 μL buffer solution first, and fluorescence spectra were obtained. BSA stock solutions were then added to make 0.1%, 0.3% and 0.5% mixed solutions. The fluorescence background of 0.1%, 0.3% and 0.5% BSA solutions without either free or encapsulated aptamers were also acquired and subtracted.

2.6 Urine Sample Preparation

To demonstrate the resistance of the aptamer-PPN sensors to human DNase we dialyzed a urine sample using a spin tube (Vivascience, UK; Product No. VS2002) with 10 kDa MWCO membrane to remove small, potentially interfering molecules that may go across the PPN membranes. After 3 cycles of centrifugation at 4000 rpm, low molecular weight species were diluted by a factor 320. The reduced volume was compensated by nanopure water. The pH of the solution was adjusted with NaOH to between 7 and 8. Different dilutions of dialyzed urine were used to evaluate the protection provided by the liposomes: 40, 120 and 200 μL of the urine were mixed with 5 μL aptamer (free or encapsulated) in 355, 275 and 195 μL buffer solutions respectively. The final urine volume fractions were 10%, 30% and 50%.

For direct measurement of Hg$^{2+}$ in urine, no sample preparation was
performed other than pH adjustment (pH is between 7 and 8). Contaminated urine with a concentration of 100 ppb or 500 nM Hg$^{2+}$ was made by mixing 5.0 μM Hg$^{2+}$ stock solutions with collected human urine (500 nM final Hg$^{2+}$ concentration). This contaminated urine sample was then combined with free or encapsulated aptamers. The final concentration of Hg$^{2+}$ in urine and aptamer solution was 50 nM. Clean (uncontaminated) urine and free aptamers were also used in a control study, in which clean urine samples were mixed with free or encapsulated aptamers, and free aptamers were mixed with clean or contaminated urine samples.
CHAPTER 3: RESULTS AND DISCUSSION

3.1 Vesicle Size Study

The sizes of the prepared liposomes were studied by QELS. Fractions collected during the separation of the encapsulated aptamers in PPNs from free aptamers were diluted by adding 30 μL of the vesicle solution to 770 μL buffer. This diluted solution was tested by QELS (Figure 3.1).

Figure 3.1.1 Size distribution A was measured after preparation and purification of aptamer functionalized PPNs. The distribution B was obtained 24 hours later. Total scattering intensity was $7.35 \times 10^6$ and $8.64 \times 10^6$ for A and B respectively.

Figure 3.1 shows little change in size distribution after aptamer-PPNs were stored for 24 hours at 4°C. The total scattering intensity, however, increased by ca. 17%. We suspect that during storage, aptamer functionalized PPNs may start to aggregate, yielding large liposome clusters. Since clusters scatter light more than individual liposomes, the overall scattering intensity increases. The size distribution
of the liposomes after storage (Figure 3.1B) shows slightly larger sizes (600 nm vs. 500 nm) and higher proportion of larger species compared to freshly extruded liposomes (Figure 3.1A). Because the size distribution centered at 120 nm was preserved without decrease of scattering intensity and only slight aggregation was observed, we believe the liposomes were stable for over 24 hours when stored at 4°C, results that are consistent with previous studies.

3.2 Sensor Characterization

3.2.1 Calibration Curves

Hg$^{2+}$ folds the aptamer by forming T-Hg-T complex, and K$^+$ can coil the DNA by forming G-quadruplex. As more Hg$^{2+}$ or K$^+$ is added to the free aptamer, the fluorescence intensity decreases (Figure 3.2).
Figure 3.2.1 Fluorescence spectra of free aptamer with increasing Hg\textsuperscript{2+} (A) and K\textsuperscript{+} (B) concentrations.

From the calibration curves shown in Figure 3.3, it can be concluded that the aptamer is more sensitive to Hg\textsuperscript{2+} since the mercury concentration is 4 to 5 orders of magnitude lower than those of K\textsuperscript{+}. This data agrees with previous reports from Chang and coworkers.\textsuperscript{21} To generate the calibration curves the change of fluorescence ((FL\textsubscript{0}-FL)/FL\textsubscript{0}, where FL\textsubscript{0} is the initial fluorescence intensity, and FL is the intensity after addition of either Hg\textsuperscript{2+} or K\textsuperscript{+} was used as a function of the concentration of the metal ions.
Figure 3.2.2 Calibration curves for the response of free aptamer to (A) Hg$^{2+}$ and (B) K$^+$.  

The response curves for free aptamers and encapsulated aptamers in both mixed lipid liposomes and pure DOPC liposomes are shown in Figure 3.4. By comparing the response curves of free aptamers and encapsulated aptamers (1:1 mixed lipids), the two curves have overlapped error bars (standard deviations) for both Hg$^{2+}$ and K$^+$ studies, suggesting that the activity of the aptamers are not affected by encapsulation in the PPNs.
As we expected, K⁺ could not penetrate the DOPC bilayer (Figure 3.4B Red). However, Hg²⁺ could enter the lipidosome and interact with the aptamers when the concentration is greater than 100 nM. This observation is in good agreement with a previous investigation by Nakada and coworkers, where Hg²⁺ was used to permeabilize the egg PC liposomes in the presence of cholesterol. They found that at 100 nM, inorganic mercury was able to disrupt the membrane introducing porous sites. Furthermore, there is essentially no difference between the methyl mercury and Hg²⁺ regarding the permeabilization of the PC lipid bilayers. Other metal ions such as: Na⁺, K⁺, Ca²⁺, Mg²⁺, Cd²⁺, Ba²⁺Mn²⁺, Pb²⁺, Zn²⁺ and Cu²⁺ have no such effect on PC lipid bilayer. This unique property of Hg²⁺ may explain its much higher toxicity.
compared to other heavy metals.

3.2.2 Protection from Biofouling and DNAse Digestion

Fluorophores often interact with proteins such as Bovine Serum Albumin (BSA), resulting in an increase in fluorescence intensity, possibly due to interactions between the fluorophore and the hydrophobic segments of the protein.\textsuperscript{36,39,37} For free aptamers labeled with fluorescein, this effect is illustrated by the fluorescence spectra shown in Figure 3.5.
Figure 3.2.4 Fluorescence spectra of free aptamers mixed with BSA. The spectra show the fluorescent intensity of the free aptamers in the presence of 0.1% (A), 0.3% (B) and 0.5% (C) BSA. The symbol ■ indicates native fluorescence from the aptamer, while ● shows the fluorescence after mixing the BSA. A bar graph summarizes the data from the spectra. (D)

We observed that the emission intensity increased by approximately 100% for all protein concentrations once BSA was added to the free aptamer solution. This
bio-fouling effect was minimized by encapsulating the aptamer inside PPNs (Figure 3.6), which act as a size selective barrier.

Figure 3.2.5 Change of fluorescence (normalized using fluorescence intensity before addition of BSA) after adding different amounts of BSA to free aptamer (■), aptamers encapsulated in pure DOPC (▲) and aptamers encapsulated in 1:1 DOPC/BisSorbPC mixed liposomes. (■)

Figure 3.6 shows a plot of change in fluorescence intensity vs. % BSA (w/w) for free aptamers, aptamers protected in DOPC liposomes, and aptamers protected in 1:1 (DOPC: BisSorbPC) mixed lipid PPNs. Fluorescence changes in aptamer-PPNs are much smaller compared to free aptamer. At 0.1% and 0.3% BSA, the fluorescence intensity increases 6% and 8% respectively for aptamers encapsulated in both DOPC liposomes and mixed PPNs. At 0.5% BSA, a 17% fluorescence increase was observed for encapsulated aptamers; while a ca. 120% increase in emission intensity for free aptamers were measured at all 3 BSA concentrations. These results indicate that liposomes prepared from either pure DOPC or 1:1 mixed
DOPC/BisSorbPC can protect the aptamer from non-specific interactions with large proteins.

We also tested whether liposomes can protect the aptamers from DNAse digestion since DNAse can be found in biological samples such as urine. Figure 3.7 shows fluorescence spectra of free aptamers with and without Hg$^{2+}$ before and after digestion with DNAse I. An increase in fluorescence intensity was observed after digestion in the absence of Hg$^{2+}$, possibly because the DNAse degraded the aptamer in such a way that the quencher and fluorophore were separated into two different molecules. When no longer linked by the aptamer, the probability of an encounter between the two was drastically reduced. It was interesting to note that once Hg$^{2+}$ was spiked into the sample, a decrease in fluorescence intensity was observed. The aptamer showed a 17% fluorescence change when 200 nM Hg$^{2+}$ was spiked into the sample after digestion. This phenomenon suggests that some of the activity of the aptamer was retained when no protection was provided. We suspect that although the aptamers break into segments after digestion, there is a possibility that the aptamer was not completely reduced into single nucleotides. The fluorophore and the quencher may be still attached to the GGT or TGG sequences. Consequently, Hg$^{2+}$ may bring the two segments together, resulting in a decrease in fluorescence intensity. Indeed, the DNAse I we utilized yields tetranucleotides as the smallest average fragment according to the manufacturer’s specification sheets. In addition, the alien structures of fluorescein and dabcyl may hinder the digestion
process. In this case, $K^+$ could serve as a better indicator for examining the protection liposomes provide against DNAse due to the fact that the G-quadruplex requires higher structural integrity to form.

![Fluorescence spectra](image)

Figure 3.2.6 Fluorescence spectra of free aptamers before and after DNAse digestion. The fluorescence intensity of the aptamer before digestion (▲) is approximately 13k counts. The fluorescence intensity first increases after digestion (●) then decreases when 200 nM (▲) and 2200 nM Hg$^{2+}$ (*) was added.

The comparison between the activities of protected and unprotected aptamers after digestion, as measured by fluorescence intensity is shown in Figure 3.8. While free aptamer showed little response to $K^+$ addition (1.7% change in intensity) after digestion with DNAse I, encapsulated aptamers exhibited a 42% change in fluorescence intensity when $K^+$ was added to the samples.
Figure 3.2.7 Fluorescence spectra of free (A) and encapsulated aptamer (B) after digestion with DNAse I. In both plots, the initial fluorescence intensity after DNAse digestion is indicated by (■) and the fluorescence level after addition of 9.76 mM K⁺ is shown with (○).

Figure 3.2.8 Investigation of the protection provided by PPNs prepared with 1:1 mixed lipids using 9.76 mM K⁺. Free aptamers (A) show almost no activity after digestion; however the response from free aptamers without digestion (B) and response from encapsulated aptamer (C) after digestion are significantly greater.
Pure DOPC vesicles were also prepared to encapsulate aptamers as a control study because, unlike bis-SorbPC, DOPC forms nonporous liposomes that are impermeable to ions. Therefore, we expected zero activity after K$^+$ was spiked even with successful encapsulation most ion. Indeed, little response was observed after 9.76 mM K$^+$ was spiked to aptamers encapsulated in DOPC vesicles (Figure 3.10). However, after Triton X-100 was added (0.625 mM), the fluorescence intensity decreased after 10 minute incubation (Figure 3.10). The fluorescence only decreased upon addition of surfactants since K$^+$ was not accessible to the aptamers trapped inside the liposome until the lipid bilayer was disrupted.

![Bar graph showing the fluorescence of DOPC encapsulated aptamers before and after the liposomes were disrupted in the presence of 9.76 mM K$^+$.

We have demonstrated that phospholipid liposomes can protect the aptamers from DNAse digestion and biofouling. More importantly, the activity of the aptamer were also preserved and unaffected by either the encapsulation process or PPNs.
3.2.3 Utilization of Aptamer Functionalized PPN Sensors

To explore the utility of aptamer-PPN sensors, we tested the response of the sensors in purified human urine, a real world sample known to contain DNAse. To examine the protection from human DNAse while minimizing the small molecule interferences, the urine sample was dialyzed so that the low molecular weight species was diluted by a factor of 320. The dialyzed urine samples were mixed with both free and encapsulated aptamers in DOPC: bisSorbPC mixed liposomes, and then incubated for 1 hour at 37°C. Hg\(^{2+}\) or K\(^+\) was then added to test the activity. In the absence of the metal ions, we observed increased fluorescence levels from free aptamers, while the encapsulated aptamers show fairly consistent emission intensities regardless of the amount of the urine present in the mixture after incubation (Figure 3.11). We believe the nuclease in human urine degrades the free aptamers causing the increased fluorescence intensity similar to that seen in Figure 3.7 while the encapsulated aptamers acquire protection against degradation.

After either Hg\(^{2+}\) or K\(^+\) was spiked to the incubated mixture, the activity of the encapsulated aptamers, calculated as the change of fluorescence intensity, also show little variation regardless to the level of urine in the mixture. (Figure 3.12). In contrast, the response of the free aptamers decreases as urine content increases, indicating increased degradation of the aptamer become at higher urine levels.
Figure 3.2.10 Fluorescence intensities at 517 nm of free aptamer (A) and aptamer-PPNs (B) after incubation with 0%, 10%, 30% and 50% (v/v) dialyzed urine samples but without metal ions.

Figure 3.2.11 Relative change of fluorescence after spiking 9.76 mM K⁺ (A) or 1.22 μM Hg²⁺ (B) as a function of urine levels. Free aptamer response (■); encapsulated aptamer (□).

The aptamer-PPNs were further tested with unpurified urine samples. A
contaminated urine sample containing 100 ppb (500 nM) $\text{Hg}^{2+}$ was prepared by spiking $\text{Hg}^{2+}$ stock solution to the collected urine (Figure 3.13). When encapsulated aptamers were mixed with the contaminated urine, a $26\pm2\%$ fluorescence change for 50 nM $\text{Hg}^{2+}$ was observed, which is consistent with $22\pm4\%$ fluorescence intensity change from the calibration curve for encapsulated aptamers at 50 nM $\text{Hg}^{2+}$ (Figure 3.4). However, at least 50% change of fluorescence intensity was observed from the free aptamers when introduced to either clean or contaminated urine samples. Although the contaminated urine sample induced 59% fluorescence change, 8% higher than the clean urine (Figure 3.13), the response indicated an equivalent quenching effect to 100 nM $\text{Hg}^{2+}$ though only 50 nM $\text{Hg}^{2+}$ was present. Based on these observations, we concluded that the free aptamers became unreliable when directly introduced into urine samples.

It is also noted in Figure 3.1.13 (D) that the clean urine gives 8% fluorescence change, which is much lower than the contaminated urine sample with 50 nM $\text{Hg}^{2+}$. This quenching effect may arise from the presence of a small amount of $\text{K}^+$ in the sample. The $\text{K}^+$ level in human urine was thoroughly studied by Putnam. The concentration of $\text{K}^+$ varies from 0.75 g/L (19 mM) and 2.61 g/L (67 mM). After 1 to 10 dilution upon mixing with aptamer solution, final $\text{K}^+$ concentration of 1.9 mM and 6.7 mM is expected. If the quenching of clean urine completely comes from $\text{K}^+$, the $\text{K}^+$ level in the donor may have been even lower than the literature value by referring to the calibration curves. As we noticed from the calibration curve (Figure
3.9), our aptamers are much more sensitive to Hg$^{2+}$ (nM) than K$^+$ (mM). Furthermore, diet control before sample collection could greatly reduce electrolyte concentration in urine. Therefore, we believe that it is still feasible to use this system to test Hg$^{2+}$ in human urine.

![Figure 3.2.12](image)

**Figure 3.2.12** Relative change of fluorescence of free and encapsulated aptamer in the presence of 50 nM Hg$^{2+}$: free aptamer (A); encapsulated aptamer (B); encapsulated aptamer with 50 nM Hg$^{2+}$ in urine (C); encapsulated aptamer with clean urine (D); free aptamer response with 50 nM Hg$^{2+}$ in urine (E); free aptamer with clean urine (F).

3.3 Conclusions

PPNs have several promising properties: easy and mild preparation and purification, excellent resistance against protein (BSA) biofouling and nuclease digestion and a size selective membrane allowing the analytes to diffuse into PPNs and interact with the encapsulated sensing chemistry. In addition, activity towards target ions was preserved, indicating that neither encapsulation nor PPNs interferes
with the active probes. The encapsulated aptamers show resistance to non-specific interactions with BSA and degradation by DNase digestion. Free aptamers showed a 120% increase in fluorescence intensity at 0.1, 0.3 and 0.5% BSA levels (w/w), while the protected aptamers showed only 6%, 8% and 17% increase in intensity respectively. In DNase digestion experiment, free aptamers showed a 1.7% fluorescence intensity change to 9.76 mM K$^+$ after digestion indicating significant loss of activity. In contrast, the encapsulated aptamers showed 42% fluorescence change under the same conditions.

For complex real world samples, contaminated urine samples were analyzed without any preparation except pH adjustment. The contaminated samples were directly mixed with the encapsulated aptamers and fluorescence was measured. We were able to detect 50 nM (10ppb) Hg$^{2+}$ in urine samples with encapsulated aptamer sensors, below the urine mercury threshold set by BAT.$^1$ The fluorescence intensity of free aptamers was quenched by at least 50% falsely implying the presence of at least 100 nM Hg$^{2+}$. Due to minimal sample preparation, the entire analysis time is less than 20 minutes.

Although there is a potential interference from K$^+$, this particular aptamer is much more sensitive to Hg$^{2+}$, and diet control can also be applied to minimize the K$^+$ level in urine. In fact, even without any diet control, the collected urine did not show significant interference. Therefore, we believe this encapsulation system can still serve as a urine mercury sensor. Based on our data and observations, PPNs have a
great potential as a general protection/encapsulation platform for various applications such as sensing and gene/drug delivery.
CHAPTER 4: FUTURE DIRECTIONS

4.1 Porous Phospholipid Nanoshells for Serum Cocaine Sensor

PPNs proved to be a reliable protection mechanism for sensing analytes in complex sample matrices. The activity of the aptamer was well preserved; non-specific interactions with BSA and DNAse digestion were blocked by the liposomes. Furthermore, the urine sample study showed consistent responses with the calibration curve only with minimal sample preparation (pH adjustment). Conversely, the free aptamers showed poor resistance in complex samples such as urine. Based on our observations, the enhanced viability of liposome protected aptamers may be applied in other biological fluids.

Cocaine is an addictive drug that related to both acute and chronic cardiovascular diseases including acute myocardial infarction, myocardial ischemia (both silent ischemia and ischemia associated with angina), acceleration of the development of atherosclerosis, myocarditis, cardiomyopathy (both dilated and hypertrophic), arrhythmias, hypertension, aortic dissection, and endocarditis.\(^6\) Among other causes of cardiovascular disease, it is critical to recognize the cocaine related ischemia or infarction,\(^6\) since cocaine users may get harmed by certain drugs or even suffer lethal effects.\(^6\) Traditional detection method for cocaine is done by measuring the cocaine metabolites in urine samples since cocaine is extensively metabolized in human.\(^6\) Although this approach is widely used, it requires long analysis time and has false
positive possibilities since the target molecules are not directly probed. Apparently, a fast and direct measurement is desired in case of medical emergency.

Aptamers that can specifically bind cocaine have been developed and characterized by Stojanovic and coworkers. The structure of the aptamer and sensing scheme is shown in Figure 4.1

Figure 4.1.1 F7-9D indicates the aptamer for binding cocaine. “F” is the fluorescein, and “D” is the quencher Dabcyl. The cocaine binds in the 3-way junction of the aptamer, causing fluorescence quenching.

However, the author did not address the feasibility of using this aptamer to detect cocaine in human serum samples. Based on the fact nucleases are present in serum, we expect aptamer degradation and compromised activity for unprotected probes. Therefore, it is natural to extend the application of porous phospholipid vesicles to anti-cocaine aptamer sensing systems. Compared to HPLC/MS for cocaine drug detection, liposome protected aptamer sensors is more cost effective and fast as an alternative analysis method.

4.2 Polymerization/Stabilization of PPNs
Although porous phospholipid vesicles have several excellent properties, improvement can still be made. The stability of the liposomes can be enhanced by using polymerizable lipids and polymerization of the bilayer. Polymerized PPNs are expected to have much longer storage time. In addition, stabilization is critical if intracellular sensing is desired. For example, the unstabilized liposomes may fuse with the cell membrane and the protection for the aptamers or other reporting probes will be lost. The polymerization can be realized to achieve higher stability. However, radical polymerization can cause damage to the fluorophores or DNA based aptamers. A mild, efficient and fast polymerization would be ideal for this purpose.
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