Protein Affinity Extraction Of Prostate Specific Antigen (PSA) Using Submicron Spheres

Yao Wang
Purdue University

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By Yao Wang

Entitled PROTEIN AFFINITY EXTRACTION OF PROSTATE SPECIFIC ANTIGEN (PSA) USING SUBMICRON SPHERES

For the degree of Master of Science

Is approved by the final examining committee:

Mary J. Wirth

Chengde Mao

Hilkka I. Kenttämaa

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Approved by Major Professor(s): Mary J. Wirth

Approved by: R. E. Wild 4/24/2014

Head of the Graduate Program  Date
PROTEIN AFFINITY EXTRACTION OF PROSTATE SPECIFIC ANTIGEN (PSA)
USING SUBMICRON SPHERES

A Thesis
Submitted to the Faculty
of
Purdue University
by
Yao Wang

In Partial Fulfillment of the
Requirements of the Degree
of
Master of Science

May 2014
Purdue University
West Lafayette, Indiana
To  

Rongjun Wang and Fengying He, my parents  
and  
Lvdai He and Daifu Lei, my grandparents
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ABSTRACT

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PSA has been used as a biomarker for prostate cancer for a long time. To characterize different glycoforms of PSA using techniques like UHPLC and cIEF, concentration and purification of PSA from complex samples becomes necessary. Our group has developed submicron silica spheres based affinity beads to extract PSA through immunoprecipitation. This study focuses on measuring the bead performance when antibody type/amount was varied and PSA concentration was lowered for clinical use. To better satisfy the requirement of large-scale extraction, several attempts were made to increase the bead capacity. The results will be presented and future directions of the project will be discussed.
CHAPTER 1 INTRODUCTION

1.1PSA Test Overview

Early detection of cancer saves life. Current treatment strategies have higher probabilities to cure with earlier detection. However, conventional diagnostic methods such as mammography have not reached the expectation of sensitivity and specificity for detecting cancer at early stages. Biomarker is another important tool for detection and it seems more potential since it detects diseases in a molecular level. Possible biomarkers include mutated genes, abnormal amount of proteins, altered post-transcriptional modification\(^1\). The discovery that free DNA was present in the serum of cancer patients decades ago opened the era of serum test\(^2\).

One of the established tests is the prostate specific antigen (PSA) test for prostate cancer. PSA is a glycoprotein produced predominantly by the prostate gland. It exists in the seminal fluid in an enzymatic active form and in the blood in three molecular forms: complexed PSA (cPSA) in which PSA covalently bound with \(\alpha1\)-antichymotrypsin (ACT), non-complexed free PSA (fPSA) and active PSA entrapped by \(\alpha\)-macroglobulins (\(\alpha2\)-M) and pregnancy zone protein (PZP)\(^3\). The sum of fPSA and cPSA in serum is referred to as total PSA (tPSA). Moreover, some Pro or precursor forms of PSA (pPSA) in serum like [-2]proPSA are considered as potential biomarkers for prostate cancer. The PSA test measures the level of PSA in a man’s blood\(^4\). In the past, PSA levels of 4 ng/ml
or lower was considered normal while a man with a PSA level higher than 10 ng/ml was recommended a prostate biopsy to see whether prostate cancer was present. However, this method lacks specificity because an elevation in PSA can be caused by not only prostate cancer, but also a number of benign prostate conditions including prostatitis (inflammation of the prostate) and benign prostatic hyperplasia (BPH). To make better use of PSA to differentiate prostate cancer and benign conditions, researchers have investigated other aspects of PSA including fPSA percentage in tPSA, Prostate Health Index (Phi, a parameter combining tPSA, fPSA and [-2]proPSA), PSA density of the transition zone, age-specific PSA reference ranges and PSA level change velocity. However, these methods fail to allow clear differentiation when PSA level is in the gray zone—between 4 and 10 ng/ml. Recently, more and more research has been focused on the carbohydrate structure of PSA because there is evidence that the cellular glycosylation process, especially sialylation, is perturbed in tumors, which might help in differentiating cancer from normal cells. For example, free PSA in serum from prostate cancer has been found to have a lower α2,6-linked sialic acid and higher α2,3-linked sialic acid than those from BPH.

In general, the analysis of PSA involves two steps: purification and characterization. Purification methods include chromatographic techniques that remove albumin and immunoglobulin from serum, and immunoprecipitation (IP) in which a protein (antigen) is precipitated out of solution using its antibody which can specifically bind to the particular protein. Characterization methods include UHPLC, mass spectrometry, electrophoresis and western blotting. Some other characterization methods like immunoassays have no or not strong requirements on prior purification because they are
based on the highly specific interactions (e.g., antibody-antigen interaction). Traditional immunoassays include enzyme-linked immunosorbent assay (ELISA) \textsuperscript{17, 18}, fluoroimmunoassay (FIA) \textsuperscript{19}, electrochemiluminescence immunoassay (ECLIA) \textsuperscript{20}, metalloimmunoassay \textsuperscript{21} and glycosylation immunosorbent assay (GISA) \textsuperscript{12}. Current research on immunoassays has been focused on increasing efficiency by utilizing biofunctionalized nanoparticles (NPs). An example of such methods is the electrochemical immunoassay (EIA) which measures the changes in light, current, potential, capacitance and conductivity upon immunocomplex formation on the electrode surface \textsuperscript{22}.

While immunoassays have the convenience of unnecessary purification and are reaching lower and lower limit of detection (LOD) \textsuperscript{3}, most of them are focused on the quantification but are not informative of different glycosylation forms of PSA. Even though techniques like GISA are using different lectins to recognize different carbohydrate structures \textsuperscript{12}, it is neither efficient nor practical to differentiate each of the ~40 glycoforms \textsuperscript{23} of PSA in this way. On the other hand, techniques like UHPLC and mass spectrometry are getting more and more powerful in separating these glycoforms for either digested or undigested PSA \textsuperscript{13}. To characterize PSA using these methods, our work is focused on purifying PSA using IP. This technique can be used to concentrate and isolate a protein from a complex sample, like serum, which contains thousands of different proteins.
In general, it can work in either direct way or indirect way:

1) Direct capture: To capture the target protein, the corresponding antibody is immobilized on a solid-phase substrate. Then the substrate with bound antibodies is added to the protein mixture and the target protein is captured onto the substrate via the antibody.

2) Indirect capture: The antibody is added directly to the protein mixture to bind its antigen (target protein), then the solid substrate coated in protein A/G (a type of protein that will bind the antibody) is added to the mixture and the antibody-antigen complex will stick to the substrate via the protein A/G.

After capture, the target protein is released in harsh conditions, like in low pH buffer, and is collected for future analysis. The indirect approach is preferred when the protein concentration or the affinity constant is low and when the binding kinetics of the antibody to protein is slow. In other situations, the direct approach is more commonly used. To conduct either process, the solid substrate which offers sites to bind antibodies or protein A/G becomes vital: a good substrate should have high binding capacity, low background, fast binding rate and reasonable price. Chapter 1.2 will introduce the two most commonly used commercial substrates, or to say, affinity beads.

1.2 Comparison of Commercialized Affinity Beads

Agarose bead are the most widely used solid substrate for immunoprecipitation. It possesses a sponge-like, highly porous structure endowing itself with large surface to volume ratio, thus high binding capacity. But the side effect of such high binding
capacity is when the binding sites are not saturated with antibodies they will bind anything that will stick, thus elevating the background signal. The accompanying disadvantage with the porous structure is low density which makes it hard to precipitate and results in low recovery ratio. In addition, the variable pore size imposes an upper limit that may affect the binding of extremely large protein complexes to internal binding sites.

A much newer alternative to agarose bead is the magnetic bead. Unlike the former, magnetic beads are solid spheres where antibodies can only bind to the surface, thus there is no limit on protein size. To compensate for the loss of capacity, magnetic beads usually have smaller size (1-4μm) than agarose beads (50-150μm). The highlight of this bead is the superparamagnetism that makes the separation of beads from solution easier without vigorous centrifugation, thus helping maintain the structure and function of proteins. But the accompanying disadvantage is the high cost. Also, there is still space to lower bead size to reach higher capacity.

The comparison of the two beads is also listed in Table 1.1.

1.3 Development of Sub-Micron Silica Affinity Beads

Our former group members have successfully designed a type of sub-micron silica spheres for immunoprecipitation/affinity extraction. We chose silica because 1) it is nontoxic, 2) its surface allows easy modification and 3) monodisperse silica particles with different size can be purchased. Basically, the silica spheres are coated with a mixed self-assembled monolayer, initiated by which a second layer of epoxy-activated
polyacrylamide has grown on the silica surface\textsuperscript{13} using atom transfer radical polymerization (ATRP) (Scheme 1.1a). The epoxy groups on the bead surface will react with the amino groups in the antibody (from either the N-terminal or lysine residue) so that the antibody can bind to the bead covalently without eluting with antigen in the final step (Scheme 1.1b). The polyacrylamide is highly hydrophilic thus can minimize non-specific binding of proteins. Besides, it forms a brush layer that has been designed to be closely spaced to sterically exclude proteins hence protein-silanol interactions are avoided\textsuperscript{24}. Our products try to beat commercial products in binding capacity by using small size (350 nm) particles. Compared with agarose, the solid sphere allows it to precipitate completely after centrifugation to reduce sample loss; compared with magnetic beads, silica is a common material and won’t cost much.

So far the beads have been proved to be able to extract PSA without releasing antibody and minimize non-specific binding with the highly hydrophilic surface. However, the highest capacity is around 100ng PSA/mg beads, which has no advantage against commercial products. By increasing the capacity, more target proteins could be extracted using fewer beads, thus lowering the cost. Chapter 1.4 will discuss possible ways to increase bead capacity. Besides capacity which is measured when PSA is in excess, it is also a big concern how beads will perform when PSA is limited/of low concentration because in clinic use a man’s PSA level in blood is less than 1000 ng/ml\textsuperscript{25}. Whether the recovery ability will keep constant in different concentrations will be answered in Chapter 4.
1.4 Possible Ways to Increase Bead Capacity

The bead capacity might be increased in the following ways:

The reaction conditions for affinity extraction are not fully optimized: the original time scale for each step is around 24h, which might lead to the loss of antibody activity. But inadequate incubation might result in an incomplete reaction either between beads and antibodies, or between antibodies and antigens. So a series of trials having different incubation time can be conducted to find the optimal time at each step. Besides, the fastest rate between the reaction of epoxy group and protein occurs at pH over 8 rather than 7 (what is currently used), which can help reaching the maximum capacity in shorter time. But higher pH is also suspicious of harming the antibody activity. So a combination of different pH and incubation time needs to be optimized for the first step (antibodies bind to beads).

The Fab region of antibody has been blocked: Each antibody consists of two heavy chains and two light chains joined to form a “Y” shaped molecule (Scheme 1.2). The arms of the Y contain the sites that can bind two antigens, thus are called Fab region. The base of the Y consists of several constant domains pertaining to a particular type of antibodies and is called Fc region. The epoxy groups randomly react with the amino groups, both in the Fab and Fc region of the antibody since both of them could contain lysine (Scheme 1.3a). If the amino group in the Fab region is reacted, the antigen binding sites will be blocked and cannot be exposed to target protein. In turn, the bead capacity will be decreased. A possible solution is to attach protein G, an Fc binding protein, to the bead surface first, then use the system to attach antibody (Scheme 1.3b). But the problem
with this solution is that the interaction between protein G and antibody is not covalent so that antibody will be eluted when antigen is eluted. To solve this, crosslinking reagent can be added to covalently link Fc region and protein G\textsuperscript{27}. But the reaction needs to be optimized so that the structure of Fab region will not be altered.

The ratio of functional groups on bead surface is not fully optimized: the bead surface with a higher ratio of epoxy groups is more likely to react with antibodies, but also will become more hydrophobic. The hydrophobic surface is not favorable because it will increase the non-specific binding, thus lower the signal-to-noise ratio. In addition, it makes the beads hard to disperse and easy to agglomerate, thus exposing less surface area to react. So beads modified with different ratio of monomers can be compared to find the optimal ratio of functional groups.

The epoxy group might not be the most efficient group to react with amino group: Currently, more and more research labs and companies are using N-Hydroxysuccinimide (NHS) as the functional group\textsuperscript{28} (Scheme 1.4). It could potentially decrease the time for reaction and increase the capability of beads. Besides, NHS is more hydrophilic than epoxy group, thus we could increase the ratio of functional groups on the surface without concerning that the beads will get hard to separate.

1.5 Thesis Overview

The work reported in this thesis focuses on applying the sub-micron silica affinity beads to extract PSA for follow-up analysis.
The first chapter reviews the current development of PSA test for prediction of prostate cancer and introduces immunoprecipitation (IP) as a purification method. Our group has developed a submicron silica based affinity beads as the solid substrates for IP, trying to overcome the shortcomings of two commercialized products. The limitation on bead capacity is also discussed as well as possible ways to improve.

In Chapter 2, the reagents and procedures are listed and described for each step, including the preparation of surface-modified silica particles, characterization of the modified particles, extraction and detection of PSA, and extraction of model proteins.

In Chapter 3, all the results by following the procedures in Chapter 2 are presented. SEM image shows the particles are homogenous around 350 nm in diameter; IR spectra show the polymerization occurs on bead surface. Next the prepared beads are used for extracting PSA 1) in different type/concentration of anti-PSA to see how bead capacity will change accordingly, 2) in lower concentration of PSA to see whether the recovery ability will hold. Then various efforts to increase bead capacity were presented, including optimizing the parameters in extraction, attaching protein G to bead surface prior to attaching antibody, adjusting epoxy group ratio on bead surface and replacing epoxy group with NHS group.

Chapter 4 makes a conclusion on all the results presented in Chapter 3, points out any data that needs to be supplemented to draw more complete conclusions and discusses the future directions of this project.
1.6 References


### Table 1.1 Comparison of Agarose beads and magnetic beads

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<th>Appearances</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td>Agarose</td>
<td>50-150μm Sponge-like structure</td>
<td>High capacity</td>
<td>High background</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Less expensive</td>
<td>Low recovery</td>
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<td></td>
<td></td>
<td></td>
<td>Limit on protein size</td>
</tr>
<tr>
<td>Magnetic</td>
<td>1-4μm Solid sphere Mono or polydisperse</td>
<td>Less sample handling</td>
<td>Expensive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No limit on protein size</td>
<td>Low capacity</td>
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<tr>
<td></td>
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<td>Availability of automation</td>
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Scheme 1.1 Structure of silica affinity bead and its reaction with protein. a) The surface of the 350nm silica affinity beads is made of a mixed self-assembled monolayer (inner) and an epoxy-activated polyacrylamide layer (outer) (provided by Dr. Yimin Hua). b) The reaction between the epoxy group on bead surface and the amino group in the protein.
Scheme 1.2 Structure of antibody.

Scheme 1.3 Attach antibodies to the bead surface a) antibodies are covalently attached to bead surface so that Fab region could be blocked (e.g., the bottom two molecules). b) antibodies are attached to bead surface via protein G (represented by the red spots) in a non-covalent way so that Fab region won’t be blocked.
Scheme 1.4 Reaction between NHS group on the bead surface with protein.
CHAPTER 2 EXPERIMENTAL APPARATUS AND PROCEDURE

2.1 Preparation of Surface-Modified Silica Particles

2.1.1 Reagents

Silica particles (500 nm in diameter) were purchased from Fiber Optic Center, Inc. (New Bedford, MA), and calcined at 600 °C for 18 hours and then at 1050 °C for 3h. trichloromethylsilane (Gelest, Inc., Morrisville, PA), (chloromethyl)phenylethyl trichlorosilane (Gelest, Inc., Morrisville, PA), acrylamide (AAM, Sigma-Aldrich, St. Louis, MO), glycidyl methacrylate (GMA, Sigma-Aldrich, St. Louis, MO), methacrylic acid N-hydroxy succinimide ester (NHSMA, Sigma-Aldrich, St. Louis, MO), N-Succinimidyl acrylate (NHSA, Tokyo Chemical Inc., Tokyo, Japan), CuCl (99.999%; Alfa Aesar, Ward Hill, MA), CuCl₂ (99%; Acros Organics, Morris Plains, NJ), (+)-sodium L-ascorbate (Sigma-Aldrich, St. Louis, MO) were used as received.

2.1.2 Procedures

As described earlier, 5g of the freshly hydrolyzed silica particles were suspended in 300ml dry toluene solution containing 7 ml trichloromethylsilane and 1ml (chloromethyl)phenylethyl trichlorosilane and stirred under nitrogen overnight. The
reacted particles were then rinsed with dry toluene and allowed to dry in a vacuum oven at 60°C overnight. The following surface-initiated polymerization could proceed in two different ways.

The epoxy group was introduced by using Atom Transfer Radical Polymerization (ATRP): An deoxygenated solution containing AAM, GMA, CuCl, CuCl₂ and Tris (2-dimethylaminoethyl) amine (Me₆TREN) in N,N dimethylformamide (DMF) was prepared as described earlier.(CAUTION: acrylamide can be neurotoxic!) The silane-modified particles were suspended in the solution and stirred under Argon for 7h.

Although ATRP has been widely used in our lab to generate polymer layers on silica surface, it requires an inert atmosphere to protect Cu (I) catalyst and the glassware used to create such conditions is usually made in small size for lab use, which makes it inconvenient to operate and difficult to produce in large scale. Matyjaszewski’s group has reported an oxygen friendly version of ATRP, Activator Generated by Electron Transfer (AGET) ATRP, which allows ATRP to be performed in the presence of air. Recently our group member Charu Yerneni has applied it to polymerize AAM on silica surface and his results have demonstrated that a similar PAAm brush layer was generated as synthesized by the traditional ATRP method. Thus the N-hydroxysuccinimide (NHS) group was introduced to the bead surface using AGET ATRP. The surface-initiated AGET ATRP was performed as following: A solution containing AAM, NHSMA or NHSA, CuCl₂, Me₆TREN and (+)-sodium L-ascorbate in isopropanol (IPA)-H₂O (volume ratio = 1:1) was prepared. The silane-modified particles were suspended in the solution and stirred
under N₂ for 2h. The modified particles were rinsed with DMF (for ATRP) or IPA-H₂O mixture and allowed to dry in a dessicator.

2.2 Characterization of the Modified Particles

The particle size was measured using JEOL NeoScope JCM-5000 Scanning Electron Microscope (SEM). The surface modification was characterized using Bruker Tensor 37 Fourier Transform Infrared Spectroscopy (FT-IR).

2.3 Extraction of PSA

2.3.1 Reagents

Prostate specific antigen (PSA) was purchased from Lee Biosolutions (St. Louis, MO). Part of the PSA was labeled with Cy3 (GE Healthcare, UK) according to the labeling kit manual. A pair of capture and detection monoclonal antibodies for PSA was purchased from Fitzgerald (Acton, MA). Protein G was purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl pimelimidate dihydrochloride (DMP) and blocking buffer was purchased from Thermo Scientific (Rockford, IL). Phosphate buffered saline (PBS; 0.01M, pH = 7.4), borate buffer (0.05M, pH=8.5), citrate buffer (0.1M, pH = 3.1), triethanolamine buffer (TEA buffer; 200mM, pH = 8.9), ethanolamine buffer (MEA buffer; 100mM, pH = 8.9) and elution buffer (0.1 M glycine with 2M urea, pH = 2.9) were prepared for use.
2.3.2 Procedures

Scheme 2.1 illustrates the process of PSA extraction. The prepared beads were suspended in PBS buffer through sonication and vortex. Next the beads were resuspended in a diluted anti-PSA solution and incubated for reaction. Then the antibody coated beads were washed and separated from the solution through centrifuge. To block the remaining reactive sites and wash the antibodies attached to the bead surface through non-covalent binding, the beads were washed and incubated in the blocking buffer containing detergents. The washed and separated beads from last step were added to a diluted PSA solution and incubated for immunoprecipitation, after which the beads were separated and washed. Finally the beads were suspended in a small volume of citrate buffer via violent vortex to elute PSA. The beads were then centrifuged down while the citrate buffer solution containing PSA were collected for the following analysis. All the incubation steps were conducted at room temperature with slow tilt rotation.

Protein G could be introduced to the bead surface prior to the antibody\textsuperscript{4}: after the beads were suspended in PBS, they were resuspended in a diluted protein G solution and incubated for reaction. Then the protein G coated beads were washed and separated from the solution through centrifuge. To blocking the remaining reactive sites and wash the protein G attached to the bead surface through non-covalent binding, the beads were washed and incubated in the blocking buffer containing detergents. The washed and separated beads from the last step were incubated with anti-PSA, PSA and citrate buffer in turn as described in the normal extraction. To see the effect of crosslinking, after washed and separated from the anti-PSA solution, the beads were suspended in TEA buffer and mixed with a diluted TEA solution containing DMP\textsuperscript{4}. After a short incubation,
the beads were separated from the solution and washed with TEA buffer. Then they were incubated with MEA buffer to block any unreacted DMP and washed with elution buffer to remove any uncrosslinked anti-PSA. Then the beads were washed by PBS buffer and incubated with PSA and citrate buffer in turn as described before. To better support the conclusion, three control groups were also conducted by removing either anti-PSA, protein G, or both (Table 2.1).

2.4 Detection of PSA

UHPLC analysis was performed using Thermo Accela UHPLC system with absorbance detection. BEH300 C4 column (1.7μm, 2.1×50mm) from Waters (Milford, MA) was used as the analytical column. A gradient of solvent A, water with 0.1% TFA and solvent B, acetonitrile with 0.1% TFA, was used. The chromatogram was obtained by an accumulation of absorbance from 200nm to 220nm.

Fluorescence analysis was performed using NanoDrop 3300 Fluorospectrometer (Wilmington, DE).
2.5 Extraction of Model Protein to Compare Bead Capacities

2.5.1 Reagents

All the necessary reagents could be found in Chapter 2.3.1 except trypsin inhibitor which was purchased from Sigma-Aldrich (St. Louis, MO) and was labeled with Cy5 (GE Healthcare, UK) according to the labeling kit manual (labeled as Cy5-Trp).

2.5.2 Procedures

The prepared beads were suspended in PBS buffer through sonication and vortex, then resuspended in a diluted Cy5-Trp solution and incubated for reaction. Then the beads were centrifuged down and the supernatants were collected for fluorescence measurement using NanoDrop 3300 Fluorospectrometer (Wilmington, DE). The separated beads were first washed with blocking buffer and then with citrate buffer. After each wash, the supernatants were collected for fluorescence measurement.
2.6 References


Scheme 2.1 An illustration of protein (PSA) extraction process.

Table 2.1 Experimental design to see the effect of protein G and crosslinking.

<table>
<thead>
<tr>
<th>Label</th>
<th>Protein G</th>
<th>Block 1</th>
<th>Anti-PSA</th>
<th>Block 2</th>
<th>Crosslink</th>
<th>PSA</th>
</tr>
</thead>
<tbody>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
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<tr>
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<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>no Ab or ProG</td>
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<td>-</td>
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<td>+</td>
</tr>
<tr>
<td>no ProG</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
CHAPTER 3 RESULTS AND DISCUSSION

3.1 Silica Affinity Beads Characterization

The silica affinity beads were prepared from 500 nm silica particles. SEM image (Figure 3.1a) shows that the particle size was homogenous and around 350 nm in diameter, perhaps due to calcinations. The FT-IR spectra (Figure 3.1b) show that efficient surface-confined polymerization occurred, with the evidence of carbonyl stretch in the amide at 1664 cm$^{-1}$.

To see whether the beads could extract PSA via immunoprecipitation instead of non-specific binding, a control experiment was conducted in which no anti-PSA was added to the control group. The chromatogram (Figure 3.2) shows that no PSA was extracted in the control group, proving that 1) PSA was extracted through the interaction with anti-PSA and thus anti-PSA was attached to the bead surface successfully while maintaining its activity, 2) the bead surface has good hydrophilicity to resist non-specific binding.

3.2 Efficiency of Extracting PSA in Different Conditions

The efficiency of extracting PSA could be different with different antibody/antigen type/concentration. To see the effect of antibody types, a pair of capture and detection monoclonal anti-PSA was used. This pair was originally utilized for the detection of free
PSA using enzyme-linked immunosorbent assay (ELISA), in which Cat# 10-P20A (affinity constant ≈ $7.5 \times 10^9 L/M$) was the detection antibody¹ and Cat# 10-P21A (affinity constant ≈ $2.7 \times 10^{10} L/M$) was the capture antibody². The chromatogram (Figure 3.3a) shows that both types of antibodies were able to extract PSA when attached to the bead surface but the capture antibody extracted around three times as much PSA as the detection antibody did according to the peak ratio. So the following experiments were all carried out using the capture antibody (Cat# 10-P21A). The difference in PSA amount could be partially due to the difference in affinity constant. The peak shape also looks slightly different, indicating that the two antibodies have different affinity towards different PSA glycoforms³. Using the same type of antibody (Cat# 10-P21A), the amount of antibody has a linear relationship with the amount of PSA that can be captured (Figure 3.3b). When the added anti-PSA is more than 30 μg, the bead capacity (μg PSA extracted by per mg of beads) will be higher than 0.1 μg PSA/mg beads. To better satisfy the requirement of large-scale extraction, this value needs to be increased. And the effort of achieving higher capacities will be discussed in Chapter 3.3.

While bead capacity mentioned above was measured when PSA was in excess (e.g., 5 μg/ml×1 ml), in clinical use, PSA concentration in a man’s blood is less than 1000 ng/ml⁴ and the gray zone of PSA concentration—4-10 ng/ml is of more interest⁵. Having known that about 10 mg beads could extract around 0.3 μg PSA from 1ml of 5 μg/ml PSA solution by using 10 μg anti-PSA (Figure 3.3b), a series of trials were conducted to see if this efficiency still held in low PSA concentration solutions (400, 200, 100, 50, 25 ng/ml). To ensure the same bead concentration, all the extractions, if not specified, were
conducted in the same volume—1 ml, so the PSA masses were 400, 200, 100, 50, 25 ng, respectively. Unexpectedly, the efficiency drops significantly when PSA concentration is reduced to 410 ng/ml (Figure 3.4), where only 122 ng was extracted instead of 348 ng that could be extracted from 5 μg/ml solution (Table 3.1). When concentration is decreased further, the amount of extracted PSA drops as well, but the recovery ratio actually increases. For concentrations lower than 210 ng/ml, the chromatograms fail to provide measurable peaks for the extracted PSA.

To solve this, fluorescence quantification was used as an alternative in which PSA needed to be labeled with fluorescent dyes. The labeling might change the structure of PSA and in turn, affect its interaction with anti-PSA. To quantify this effect, similar concentrations of PSA and Cy3-PSA were extracted using the same amount of beads and anti-PSA. The chromatograms (Figure 3.5) and calculation results (Table 3.2) show that the labeling will weak the interaction, but a significant amount of PSA can still be extracted. So similarly, a series of low concentration Cy3-PSA solutions (670, 335, 167, 84, 42 ng/ml) were extracted for fluorescence detection. The recovery ratio for the 670 ng/ml trial is calculated to be 0.176, which is close to 0.154 obtained from chromatogram peak, thus strengthening the reliability of our data. When concentration is decreased to 335 and 167 ng/ml, the amount of extracted Cy3-PSA decreases but the recovery ratio increases, showing the same trend as the non-labeled PSA does. When the concentration is further decreased, the recovery ratio becomes abnormal (>1), a possible reason is that: when the concentration becomes small, the fluorescence intensity is too small to be measured; thus the solvent of solutions containing extracted PSA was evaporated through dry vacuuming and the protein was resuspended in a small volume (<5 μL) for
fluorescence measurement; the volume is so small that ±1 μL error can cause >20% error in fluorescence intensities. In addition, to prove that the beads extract PSA via anti-PSA instead of non-specific binding, a control group with no anti-PSA added should be conducted.

3.3 Efforts to Increase Capacity

3.3.1 Optimize Extraction Conditions

During the attachment of antibody, an important parameter is pH. As reported, the fastest rate between the reaction of epoxy group and protein occurs at pH over 8.6 rather than 7 (what we commonly used). But higher pH is also suspicious of harming the antibody activity. The chromatograms (Fig.3.6) show that when incubating beads with anti-PSA in two different pH buffers, the variation of bead capacity along with incubation time shares a similar pattern, which can be further proved by the plot of capacity against incubation time (Fig.3.7). But no matter how long the incubation time is, the capacity is always higher when incubating in PBS buffer (pH = 7.4) than in borate buffer (pH = 8.5), which indicates that even epoxy group may have a higher reaction activity at higher pH, the antibody works better at a more neutral pH.

So far the pH for incubating with anti-PSA has been established, the effect of time for each step needs to be characterized. Generally speaking, with longer time, the reactions are closer to completion; but the activity of antibody might be lost as well. When incubating beads with anti-PSA for different time, the eluted PSA all have similar peak
shapes in the chromatogram (Figure 3.8a) and the capacity of beads gradually increases with the increase of time (Figure 3.8b). This appears controversial with Figure 3.7, where the maximum capacity occurs at 16 h. This can partially owe to detection error of UHPLC at such a tiny scale. Besides, when beads were incubated with blocking buffer or with PSA for different time, the peaks have similar shapes (Figure 3.8c, e). The trends of capacity change (Figure 3.8d, f) seem difficult to interpret partially due to detection error of HPLC, but both plots show the maximum occur at 24 h. The three time effects all indicate that a larger capacity occurs with a longer time scale and the maximum capacity might be beyond 24 h.

3.3.2 Attach Protein G to Bead Surface Prior to Antibody

Figure 3.9 shows the effect of attaching Protein G on bead capacity. When the beads are attached with protein G prior to anti-PSA without crosslinking (the blue line), there is PSA extracted, but the amount is less than what is extracted when only anti-PSA is attached (the red line) by comparing the peak areas. This can be explained by the loss of activity when Protein G is attached to the bead surface. But what is unexpected is that no anti-PSA is eluted together with PSA as the interaction between Protein G and anti-PSA is non-covalent. More data are needed to explain this phenomenon. In fact, crosslinking reagent (in this case, DMP) is used to prevent the elution of anti-PSA by covalently linking Protein G and anti-PSA\(^7\). But no PSA is extracted when DMP is added (the black line), probably because the amino groups within Fab region of anti-PSA reacted with DMP, thus the structure got changed. The rest three trials are control groups: by comparing the pink line (neither anti-PSA nor Protein G was added) with the blue line, it
indicates that PSA is extracted not through the non-specific binding with the bead
surface; by comparing the green line (no anti-PSA was added) with the blue line, it
indicates that PSA is extracted not through the interaction with Protein G; by comparing
the brown line (no Protein G was added) with the blue line, it indicates that anti-PSA is
attached through the interaction with Protein G. In a conclusion, PSA is extracted through
the bead_Protein G_anti-PSA_PSA interaction.

3.3.3 Adjust Epoxy Group Ratio

To see whether a lower ratio of epoxy groups on the surface could increase the bead
capacity by improving its hydrophilicity, the monomer ratio of GMA and AAM was
decreased to 1:40 (1:40 beads) and compared with the previous ratio of 1:20 (1:20 beads).
The chromatograms (Figure 3.10) show that 1:20 beads have a larger capacity through
the comparison of peak areas. To find the optimal ratio of monomers, more experimental
data are needed.

3.3.4 Replace Epoxy Group with NHS Group

To see whether NHS group could increase the bead capacity, GMA was replaced with
NHSA or NHSMA, both of which contain NHS group (Scheme 3.2). To see whether they
could copolymerize with AAM on the bead surface, the monomer ratio NHSMA/NHSA
to AAM was set to be 1 to 1 at first. The FT-IR spectra (Figure 3.11) show that different
from pure AAM polymerization, the beads that were modified with mixed monomers
have triplet within 1700-1850 cm⁻¹, probably due to three different carbonyl stretches in
NHSMA/NHSA, thus indicating the successful copolymerization on the bead surface. In addition, the triplet for NHSMA is much higher than that for NHSA, indicating more NHS groups on bead surface.

To compare the binding capacity of the NHS modified beads relative to epoxy modified ones, four types of beads (reacting with GMA and AAM, labeled GMA; reacting with NHSA and AAM, labeled as NHSA; reacting with NHSMA and AAM, labeled as NHSMA; reacting with only AAM, labeled as AAM) were used to extract a model protein—Cy5 labeled trypsin inhibitor (Cy5-Trp) from the same concentration of solution. After extraction, the suspensions were centrifuged and the supernatants were subjected to fluorescence detection. AAM has the largest fluorescent intensity because there is no functional group reacting with protein and it has the best hydrophilicity (Figure 3.12a). NHSA has almost no fluorescent intensity indicating that nearly all the proteins are attached to the bead surface, but the way of binding (covalent or non-covalent) remains unknown. This can be answered by Figure 3.12b and 3.12c, where the fluorescent intensities of supernatants are still low after washing with blocking buffer and citrate buffer (pH=3.10), indicating most of the proteins are attached to NHSA beads in a covalent way. Interestingly, GMA beads and NHSMA beads don’t bind as much proteins as NHSA beads do (Figure 3.12a). Especially by comparing NHSMA beads and NHSA beads, this seems contradictory with the spectra in Figure 3.11 where the conclusion is made that NHSMA beads have more NHS groups on beads surface than NHSA beads. However, this can be explained by SN2 reaction mechanism: the reaction between NHS group and amino group happens when N in –NH₂ attacks carbonyl group in NHSMA/NHSA monomer; because of the steric hindrance effect of the extra methyl group, the
reaction between NHSMA and protein is much slower than between NHSA and protein. Later, quite an amount of the protein was washed out with blocking buffer, indicating the significant existence of non-specific binding; quite amount of protein was washed out with citrate buffer for GMA beads, indicating the covalent binding between beads and protein is vulnerable to low pH. This could explain the peaks occurring one minute later than the PSA in some of the previous chromatograms (e.g., Figure 3.2): they are very likely to be anti-PSA, part of which eluted with PSA in the low pH condition. The strong binding ability, low non-specific binding and stable covalent interaction of NHSA bead make it very promising in increasing bead performance.
3.4 References


Scheme 3.1 Crosslinking reaction between Protein G and Anti-PSA

Scheme 3.2 Molecular formula of a) NHSA b)NHSMA. c) Reaction between NHS group on the bead surface with protein.
Figure 3.1 a) SEM image of surface-modified silica beads. Bead size shrank from 500 nm to around 350 nm after calcinations. b) FT-IR spectra of 350 nm silica beads modified with copolymerization of AAM and GMA.

Figure 3.2 Chromatogram of PSA extracted from 5μg/ml solution. Black line indicates the normal operation while the red line indicates no anti-PSA was added in the previous step.
Figure 3.3 The effect of anti-PSA on the amount of extracted PSA. a) The chromatograms of extracted PSA by using different types of anti-PSA. b) The amount of Anti-PSA varies proportional to the amount of anti-PSA added by using 10.5 mg silica affinity beads (provided by Dr. Yimin Hua).

Figure 3.4 Chromatograms of PSA extracted from different concentrations of PSA
Table 3.1 Amount of PSA extracted from different concentrations of PSA and their corresponding recovery ratio calculated from the chromatogram peaks

<table>
<thead>
<tr>
<th>PSA (ng) in 1ml PBS</th>
<th>Recovered PSA (ng)</th>
<th>Recovery ratio</th>
</tr>
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<tr>
<td>205</td>
<td>86</td>
<td>0.420</td>
</tr>
<tr>
<td>410</td>
<td>122</td>
<td>0.298</td>
</tr>
<tr>
<td>5000</td>
<td>348</td>
<td>0.070</td>
</tr>
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</table>

Figure 3.5 Chromatograms of a) non-labeled PSA extracted from its 410 ng/ml solution b) Cy3-PSA extracted from its 670 ng/ml solution (The two runs were conducted in two separate days, so the baseline looked quite different)

Table 3.2 Amount of PSA extracted from labeled and non-labeled PSA and their corresponding recovery ratio calculated from the chromatogram peaks

<table>
<thead>
<tr>
<th>PSA in 1ml PBS (ng)</th>
<th>Recovered PSA (ng)</th>
<th>Recovery ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>410 (PSA)</td>
<td>122</td>
<td>0.298</td>
</tr>
<tr>
<td>670 (Cy3-PSA)</td>
<td>103</td>
<td>0.154</td>
</tr>
</tbody>
</table>
Table 3.3 Amount of Cy3 labeled PSA extracted from different concentrations of Cy3-PSA and their corresponding recovery ratio calculated from the fluorescence intensities. For the first two rows, the fluorescence intensities were measured directly from the 25μL citrate buffer solution containing eluted PSA; for the last three rows in the red box, as the concentrations were two low to be measured, the solutions were concentrated from 25μL to 3μL for measurement. The recovery ratio which was larger than one might be due to the mishandling with volume, which was too little to cause big errors.

<table>
<thead>
<tr>
<th>Cy3-PSA (ng) in 1ml PBS</th>
<th>Recovered PSA (ng)</th>
<th>Recovery ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>670</td>
<td>118.1</td>
<td>0.176</td>
</tr>
<tr>
<td>335</td>
<td>103.7</td>
<td>0.309</td>
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<td>167</td>
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<td>84</td>
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<td>1.88</td>
</tr>
<tr>
<td>42</td>
<td>60.0</td>
<td>1.43</td>
</tr>
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</table>

Figure 3.6 Chromatograms of extracted PSA when particles were reacted with anti-PSA in different pH and different time (A) Beads were reacted with anti-PSA in borate buffer (pH =8.5) for 8h, 16h and 24h, respectively. (B) Beads were reacted in PBS buffer (pH=7.4) for 8h, 16h and 24h, respectively.
Figure 3.7 Comparisons of bead capacities (μg of PSA that could be extracted by per mg of beads) when reacted with anti-PSA in different pH and time. The black spots indicate bead capacity when reacted in borate buffer (pH = 8.5) while the red spots indicate bead capacity reacted in PBS buffer (pH = 7.4). The data was calculated from the peak areas from Figure 3.6.
Figure 3.8 The effect of reaction time on bead capacities a), c), e) are chromatograms showing effect of antibody incubation time, blocking time and antigen incubation time, respectively. b), d), f) show the corresponding bead capacities calculated from peak areas.
Figure 3.9 Chromatograms showing the effect of Protein G on bead capacity. Red line (previous) represents the extracted PSA when using the previous method, that is, first attaching antibody, next blocking, then capturing PSA; blue line (no crosslink) is different from the red one in that Protein G attachment and blocking were carried before antibody and PSA interaction; black line (crosslink) is different from the blue one in that antibody was cross linked with Protein G before interacting with PSA; the rest three lines were the same as the blue line (no crosslink) except that no antibody was added (green line, no Ab), no Protein G was added (brown line, no ProG) or neither antibody nor Protein G were added (pink line, no Ab or ProG).
Figure 3.10 Chromatograms of extracted PSA using beads modified with different ratio of monomers. For the black line, GMA : AAM = 1:20; for the red line, GMA : AAM = 1:40.

Figure 3.11 FT-IR spectra of 350 nm silica particles modified with poly-AAM (black line), copolymerization of NHSMA to AAM (1 to 1 ratio, red line) and copolymerization of NHSA to AAM (1 to 1 ratio, blue line)
Figure 3.12 Fluorescence intensities of supernatants a) after the beads were incubated with Cy5-Trp, b) after the beads were washed with blocking buffer, c) after the beads were washed with citrate buffer. The three steps were conducted in order. For the black line, beads modified with GMA and AAM were used; for the red line, beads modified with NHSA and AAM were used; for the blue line, beads modified with NHSMA and AAM were used; for the green line, beads modified with AAM were used. d) Photograph of four beads and their supernatants after the final wash with citrate buffer.
CHAPTER 4 CONCLUSION AND FUTURE DIRECTIONS

4.1 Conclusion

We have prepared a type of silica based affinity beads with homogenous size of 350 nm in diameter and with a layer of epoxy-activated polyacrylamide on the surface. The beads were able to extract PSA from solution via anti-PSA on the surface while non-specific binding was negligible due to the highly hydrophilic surface. The amount of PSA that could be captured depends on not only the type but also the amount of anti-PSA. In addition, the amount of PSA is proportional to the amount of anti-PSA. When the concentration of PSA is decreased, the amount that can be recovered decreases accordingly, but the recovery ratio increases in the observable range.

To increase the capacity of beads, first the extraction conditions were optimized: it was found that a neutral pH in the step of attaching anti-PSA corresponds to a higher bead capacity than a higher pH does; among all the time scales that have been tried, the longest time (24 h) in each step corresponds to the highest bead capacity, but it remains unknown if capacity will keep increasing when the incubation time is longer than 24 h. Next, protein G was introduced to the system to see if it could help increase the bead capacity. The experiment results show that protein G does its job during the process, but decreases (instead of increases) the bead capacity. Crosslinking, on the other hand, leads to no PSA captured probably by eliminating the activity of anti-PSA. Then, when the
epoxy group ratio on the bead surface was decreased, bead capacity decreased as well, probably due to insufficient epoxy groups to act with. Finally, the epoxy group was replaced with NHS group. The preliminary experiments with model proteins show that beads modified with NHS group have a higher bead capacity through covalent binding than those modified with epoxy group. But whether it works for affinity extraction remains unknown.

4.2 Future Directions

To make the conclusions in Chapter 4.1 more solid, more data are needed:

1) Since PSA extracted from low concentration solutions cannot be measured using either UHPLC or fluorescence, ELISA can be used as an alternative because of its low detection limit.

2) More data is needed to explain why there is no anti-PSA eluted together with PSA when protein G is applied. If the interaction between anti-PSA and protein G is strong enough to resist the low pH, then crosslinking reagent becomes unnecessary.

3) More data is needed to find the optimal ratio of GMA to AAM ratio, which can be either smaller than or larger than the current one (1:20).

4) Apply the beads modified with NHSA/AAM for affinity extraction to see whether PSA could be captured. The ratio of NHSA to AAM can also be optimized because too dense NHS groups on the bead surface might change the structure of anti-PSA and affect its activity.
Besides, there are also some new directions worthy of trying:

1) Indirect approach for immunoprecipitation\textsuperscript{1}: In the context the direct approach is used, in which the antibodies are immobilized on the bead first and then the beads with bound antibodies are used to extract proteins. However, when the protein concentration is low, the indirect approach might be more effective: the antibodies are added to the target protein solution to form antibody-antigen complex; then the beads coated in protein G are used to extract the complex.

2) Combined with cIEF: our ultimate goal is to analyze the PSA in human blood to predict prostate cancer, so it is necessary to combine the immunoprecipitation step with powerful analyzing techniques. Recently, Brian Wei in our group has applied a capillary based isoelectric focusing (cIEF) technique\textsuperscript{2} to separate different glycosylation forms of PSA. So the idea is: first extract PSA from human serum using our affinity beads, and then analyze it using cIEF. But the challenge is— for the current gray zone of PSA concentration, 4 – 10 ng/ml, and under the volume limit of blood sample we can take each time, how can we extract PSA with minimum sample loss, label it and inject it into the capillary.

3) Combining with other affinity ligands: Besides anti-PSA, the affinity beads can be coated with other antibodies to capture corresponding target proteins. Besides antibodies, the affinity beads can be coated with lectins\textsuperscript{3}—proteins which can bind specific carbohydrate molecules. Therefore the coated beads can be used to separate glycoproteins from non-glycosylated proteins, or one glycoform from another glycoform. The second application can be very meaningful when studying different glycoforms of PSA\textsuperscript{4}. Furthermore, metal ions can be
introduced to the bead surface to purify specific proteins: cobalt, nickel and copper for the purification of histidine containing proteins or peptides; iron, zinc and gallium for the purification of phosphorylated proteins or peptides\textsuperscript{5}. This technique can be extended to proteins having no affinity for metal ions by genetically modifying them\textsuperscript{6}.
4.3 References


