Development of Aptamer-Coated Beads for PDGF-BB Capture

Pimchanok Sangkeaw, Jittraporn Saengkaew and Boonchoy Soontornworajit*

Department of Chemistry, Faculty of Science and Technology, Thammasat University, Rangsit Centre, Khlong Nueng, Khlong Luang, Pathum Thani 12120

Abstract

Nowadays, proteins play significant role in the advance of human well-being due to their molecular functions. Therefore, several attentions have been drawn to improve protein production including purification process. This research aimed to improve a protein purification tool for platelet-derived growth factor BB (PDGF-BB) by developing aptamer coated polystyrene beads. The aptamer was a binding site for the proteins. The aptamer was immobilized onto the beads *via* amide bond couplingstrategy that performed by using EDC. The aptamer immobilization was confirmed by flow cytometry. The effects of EDC, aptamer concentration, and acidic/basic condition on the coupling efficiency were studied. For the EDC-based coupling, the results showed that the coupling reaction increased its efficacy at high concentration of those reagents under acidic condition. After the immobilization, the capturing ability of aptamer-coated beads to PDGF-BB was tested. The amount of PDGF-BB that has been captured by the particles and eluted by complementary oligonucleotide was determined by ELISA. Results demonstrated that the aptamer-coated beads were able to capture PDGF-BB. However, the PDGF-BB elution by complementary oligonucleotide was not successful. It suggested that an elution process needed further improvements.

Keywords: PDGF-BB; aptamer; amide coupling; polystyrene beads; protein purification

1. Introduction

Proteins played a vital role in many biological processes and therefore were promising molecules to be used in a variety of application including, nutrition supplements, drugs, immunized vaccines, and diagnostic agents (Navarro *et al.*, 2009). Bioactivities of the protein conventionally came from the protein structures which presented in three dimensional structures. Consequently, effective productions that could maintain the functional structure of these bioactive molecules were main research focuses. The achievement of protein production could facilitate the well-being of our society. In general, protein production steps included protein generation from its source, protein extraction/separation, and protein purification. However, the separation and purification steps

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have been limited by conditions for maintaining the functional structure of proteins such as temperature, pH, solvents and ionic strength. The functional structure governed by the stability of the proteins depended on the protein environments including production system and also the application sites. For instance, most of the proteins were degraded in body fluid (Tessmar and Gopferich, 2007). In addition, protein purity could affect the further protein application. For example the biosensor demanded a high purity antibody (Sheng and Kong, 2012). Therefore, it was necessary to develop a platform that can separate and purify the proteins without the loss of their bioactivities.

Nowadays, there were many purification techniques that have been used in both research and industrial scales. Their strategies relied on chromatography and non-chromatographic methods such as membrane separation, crystallization and aqueous two-phase systems. These techniques demonstrated several advantages (Low *et al.*, 2007). However chromatographic techniques have suffered from size and density of the particles that govern the flow rate of the systems. Especially, the process dealing with cell culture sources was obstructed from the fouling of the adsorbent due to binding of cells and cell debris (Blank *et al.*, 2001). Membrane separation has been limited by the fouling issues and the specificity of the separated molecules (Barnard *et al.*, 2014). Whereas the usages of aqueous two-phase systems have been obstructed by specific partitioning principle for each compound, therefore it was necessary to pre-verify for a particular molecule (Salgado *et al.*, 2008).

Recently, a number of polymeric beads used for capturing target proteins has been developed and improved their preparation strategies and also their functions as summarized as follows. For instance, a bead composed of core of Fe_3O_4, a sandwiched layer of poly(4-vinyl pyridine) (4-VP) and a shell of crosslinked poly(N-isopropylacrylamide) (PNIPAM) exhibited excellent binding properties for bovine serum albumin (BSA). Its binding capability relied on temperature changes and was also reversible (Zhang *et al.*, 2016). Polypyrrole-coated magnetite nanoparticles have been prepared by ultrasonic-microwave technique. They demonstrated promising characteristics on extraction of vitamin D from milk samples (Jiao *et al.*, 2016). Molecularly imprinted core-shell microparticles that were able to bind porcine serum albumin (PSA) via a chelation of Cu(II) ions was developed based on radical polymerization of 2-hydroxyethyl methacrylate and methacrylic acid (Li *et al.*, 2016). However, the binding interaction was still relied on hydrophobic and electrostatic interaction.

The affinity interaction between specific ligands and their target proteins has been used in a variety of alternative recovery operations to succeed the separation specificity. The ligands have been tethered and/or incorporated to several materials such as membranes, columns, polymeric beads and magnetic beads (Meyer *et al.*, 2001; Franzreb *et al.*, 2006; Zhou and Tressel, 2006). These materials demonstrated a

good efficiency in protein purification based on a strong binding interaction between the ligands and the targets. There was a dilemma in this aspect because a strong binding interaction made the bonded molecule to be difficult to elute. For affinity based purification techniques, many elution processes were dealt with the harsh conditions (e.g. strong acidic and basic condition, high temperature, and un-preferable ionic strength) in order to compromise the strong interaction (Hudson, 1999; Hermeling *et al.*, 2004; Wu and Senter, 2005). Consequently the target proteins were denatured and lost their bioactivity. In addition, a number of specific ligands including peptides, metal ions, and heparin had low binding, low specificity, and/or high toxicity (Sakiyama-Elbert and Hubbell, 2000; McGonigle *et al.*, 2008; Jha *et al.*, 2009). Therefore, it was highly desirable to develop a ligand that could improve the stability while remaining the high binding affinity and specificity.

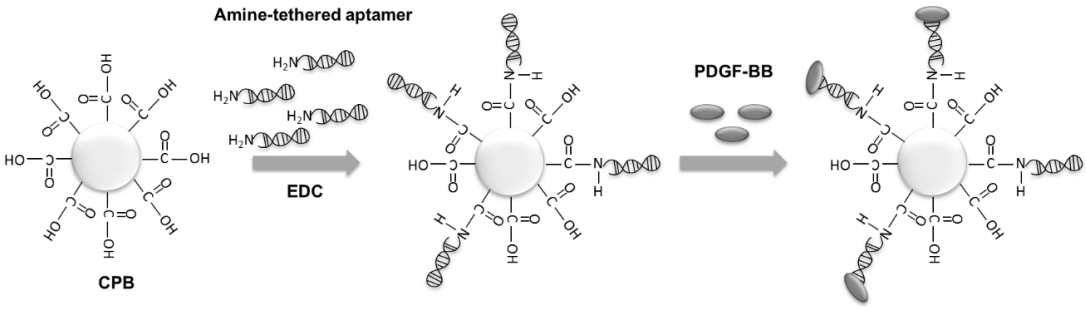
Nucleic acid aptamers were single-stranded oligonucleotides screened from DNA or RNA libraries by systematic evolution of ligands by exponential enrichment (SELEX) (Ellington and Szostak, 1990). Nucleic acid aptamers attracted significant attention in various research areas because they had unique merits as affinity molecules. In addition, they were small and stable molecules which were commercially synthesized for a variety of targets (Proske *et al.*, 2005). Because of these distinguished characteristics, aptamers were used for various biological and biomedical applications such as biomarker detection, biosensor development, and drug delivery (Savran *et al.*, 2004; Gokulrangan *et al.*, 2005; Hansen *et al.*, 2006; Li *et al.*, 2007; Cao *et al.*, 2009; Swensen *et al.*, 2009). In this study, the aptamers was integrated with polymeric beads for efficiently capturing target proteins from a solution. Aptamers could bind tightly and specifically to target proteins and could therefore be used as binding sites of protein capturing beads (Soontornworajit *et al.*, 2010). The specific interaction between PDGF-BB and the aptamer came from electrostatic forces between positive-charged amino acid and negative-charged phosphate backbone and H-Bonding between the protein and the aptamer. In addition, the binding interaction resulted in more rigidity of both molecules investigated by a computational approach (Vu, 2016). Hence, the specific binding interaction between protein and aptamer was a promising strategy in separating the target proteins from other molecules (Scheme 1).

2. Materials and Methods

2.1 Reagents

Carboxyl polystyrene beads (CPB) with an average diameter of 1.3 μm were purchased from Spherotech. Phosphate buffered saline (PBS) and *N*-ethyl-*N*-(3-diethylamino-propyl) carbodiimide (EDC) were purchased from Sigma-Aldrich. Recombinant human platelet-derived growth factor BB (PDGF-BB), and a kit for human PDGF-BB enzyme-linked immunosorbent assay (ELISA) were purchased from R & D Systems. Bovine serum albumin

(BSA) was purchased from Invitrogen. The DNA aptamer and complementary oligonucleotide were purchased from Integrated DNA Technologies. The aptamer and complementary oligonucleotide (CO) sequences were 5′-GCG ATA CTC CAC AGG CTA CGG CAC GTA GAG CAT CAC CAT GAT CCT G-3′, and 5′- TCA GGA TCA TGG TGA TGC TCT-3′, respectively. The aptamer was coupled with primary amine at 5′ terminus, and the CO was coupled with fluorescence probe at 5′ terminus.



Scheme 1 Schematic presentation of the concept.

2.2 Preparation of aptamer-coated beads

Carboxyl polystyrene beads were dispersed in PBS to a concentration of 1 mg/mL. Then 10 µL of the suspension was mixed with 2 µL of aptamer solution (100 µM). The aptamer was pre-labeled with primary amine at 5′ terminus. To form amide bond between the beads and the aptamer, EDC solution at designated concentrations was added to the suspension mixer. Aptamer concentrations were then adjusted by adding PBS. Amide coupling reaction was carried out for 30 minutes. Aptamer-coated beads were then washed by 500 µL of washing solution (PBS containing 0.05 % Tween® 20) and centrifuged down using a micro centrifuge at the speed of 13,000 rpm. The washing step was repeated 4 times for each reaction batch.

2.3 Characterization of aptamer-coated beads by flow cytometry

To confirm the success of bead preparation, 2 µg of aptamer-coated beads were treated with a solution containing 100 nM of fluorescence labeled CO for 30 minutes. Then the suspension was washed by 500 µL of the washing solution for 4 times. Fluorescence signal from the beads was determined by a BD FACS Calibur 3C/Basic flow cytometer.

2.4 Test of PDGF-BB capture and elution

For protein loading process, PDGF-BB with approximately concentration of 250 pg/mL was incubated with 2 µg of either unmodified beads or aptamer-coated beads in phosphate buffer saline (PBS) at room temperature for 30 minutes. The suspension was centrifuged to separate the beads from the

temperature for 30 minutes. The suspension was centrifuged to separate the beads from the buffer. Then the beads were suspended in a solution containing CO at designated concentration for 30 minutes. The suspension was centrifuged to separate the beads from the buffer. The amount of proteins in both the capturing and eluting buffer was quantified by ELISA.

3. Results and Discussion

This work aimed to tether PDGF-BB aptamer onto polystyrene beads using EDC as a coupling agent, which could provide an amide bond formation. Flow cytometry histogram (Figure 1A) presented a fluorescence signal from each particle sample. The fluorescence signal indicated availability of PDGF-BB aptamer immobilized onto the beads because the fluorescence signal came from the fluorescence-labeled CO which has been hybridized with the aptamer. In Figure 1A, the beads that have been treated with EDC demonstrated fluorescence shift in comparison to the control beads which have not been treated with EDC. The results clearly indicated the success of aptamer-tethered beads via amide bond formation. In addition, effect of EDC concentration on coupling efficiency was also investigated. As presented in Figure 1B, mean fluorescence intensity (MFI) of the beads that have been treated with 2.61, 26.1, and 261 mM of EDC was 4.83, 7.90, and 9.65, respectively. At increased EDC concentration, the coupling efficiency was enhanced due to the sufficient amount of reactive species. Since O-acylisourea intermediate formed by the reaction between carboxyl group and EDC was not stable in an aqueous environment, the increased amount of EDC could compensate the loss of the intermediate activity during the coupling process. However, a large amount of EDC could cause the production of N-acylurea which consequently reduced the coupling efficiency (Polyak *et al*., 2004).

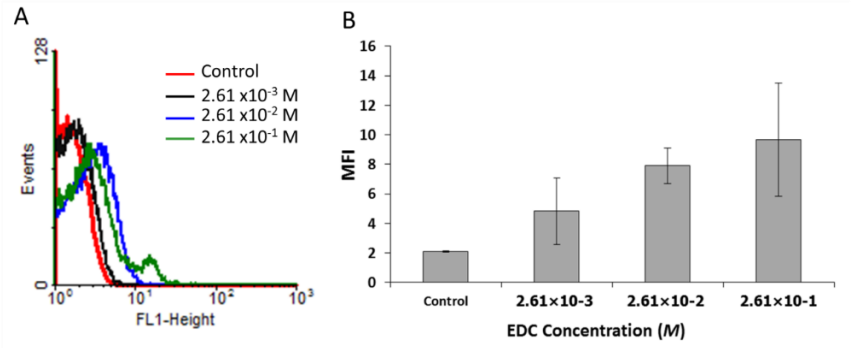


Figure 1 Effect of EDC concentration on coupling efficiency. (A) Flow cytometry histogram, and (B) mean fluorescence intensity of particles treated at designated EDC concentration. 0, 2.61, 26.1 and 261 mM.

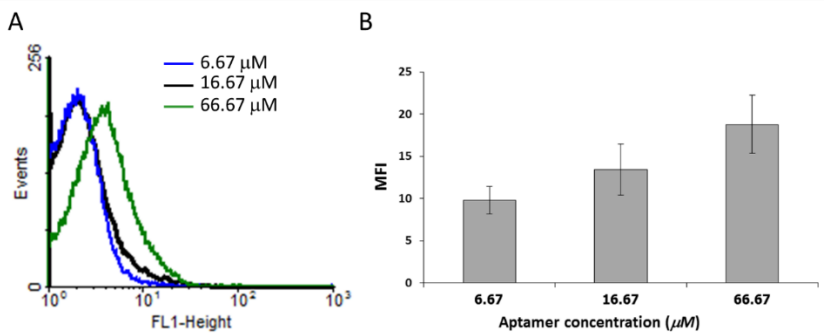


Figure 2 Effect of aptamer concentration on coupling efficiency. (A) Flow cytometry histogram, and (B) mean fluorescence intensity of the particles treated at designated aptamer concentration. 6.67, 16.67, and 66.67 µM.

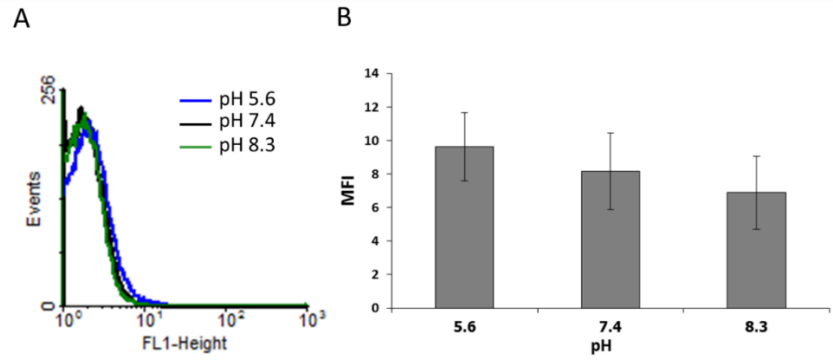


Figure 3 Effect of pH on coupling efficiency. (A) Flow cytometry histogram, and (B) mean fluorescence intensity of particles treated at designated acidic/basic condition.

Another parameter that played a role in coupling efficiency was the concentration of PDGF-BB aptamer (Figure 2). The MFI values of the beads that have been reacted with 6.67, 16.67 and 66.67 µM of the aptamer were 9.76, 13.43, and 18.75, respectively. The results demonstrated the enhancement of coupling efficiency because the increase of aptamer concentration could fundamentally increase a chance of the amino group overhung in the aptamer and the carboxyl group immobilized on the beads to react. There were rooms left on the beads for coupling reaction because MFI did not show the saturated value. It would be promising to increase the coupling efficiency by increasing the amount of the aptamer. However, using large amount of the aptamer was not practical in an economical aspect.

The experiments were further carried out to investigate the effect of pH on coupling efficiency as shown in Figure 3. The MFI values of the beads prepared in pH 5.6, 7.4, and 8.3

were 9.63, 8.17, and 6.89, respectively. The results indicated that the amide bond formation processed preferably in acidic condition, agreeing with the previous study (Polyak *et al.*, 2004). Another strategy to perform amide coupling reaction was NHS ester reaction, which was also influenced by the pH. The activation step was more efficient in mildly acidic condition (pH4.5-7.0). The amine coupling step was favorable in basic condition (pH 7.0-8.0), since the primary amine was not protonated at high pH (Polyak *et al.*, 2004).

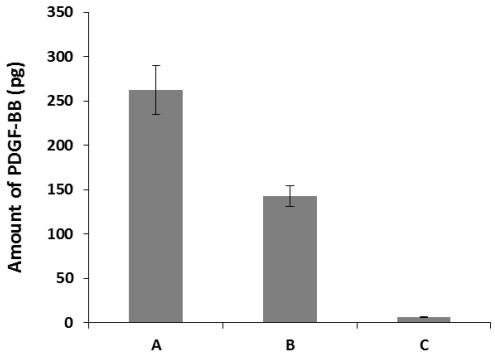


Figure 4 Amount of PDGF-BB in tested solutions. (A) Initial solution, (B) solution treated by unmodified beads, and (C) solution treated by aptamer-coated beads.

The experiment was further performed to test the hypothesis that aptamer-coated beads could capture PDG-BB in a solution. The results showed that, after incubating PDGF-BB with unmodified beads for 30 minutes, the amount of PDGF-BB left in the solution were 148 pg (Figure 4). In contrast, the amount of proteins left in solution was approximately 10 pg after the incubation of PDGF-BB with aptamer-coated beads. These results indicated that the PDGF-BB was captured on the aptamer-coated beads due to the strong binding between the aptamer and the growth factor. In principle, aptamer-protein interactions were predominated by base pairing and the resulting secondary structure of the aptamer (Zhou *et al.*, 2011). Moreover, a non-specific binding contributed partially to capturing the PDGF-BB by the beads because the amount of the growth factor left in the solution after incubating with unmodified beads was less than that in an initial PDGF-BB solution.

An efficiency of each purification technique depended on a number of parameters which were empirical for each target molecule, experimental setup, condition, and/or protocol. For chromatographic techniques, their specificity and the affinity has been improved by functionalizing the columns with ligands specifically binding to a number of antibodies such as IgG, IgM, and IgE (Sheng and Kong, 2012). The synthetic mimic ligands of proteins A and L were modified via a combination of molecular modeling and synthetic chemistry. Consequently, the capacity could be reached within a range 0.5-60 mg of antibody/g of solid support (Sheng and Kong, 2012). For membrane separation, binding ligands were either immobilized directly onto the membrane or copolymerized with monomer to form the membranes. The purification efficiency could be adjusted by optimizing the flow rate, alternating modes of flow, and coating a hydrophobic

molecule onto the membrane to reduce non-specific binding. For example, binding capacities for IgG1 and IgG2 were around 17 and 29 μg/cm^2 for dead-end mode, and cross-flow filtration, respectively (Boi, 2007). For aqueous two-phase systems, the partition efficiency for IgG could yield 93 % of purity to when the partition system composed of PEG-dextran and PEG-COOH. By altering formulation of partition components (e.g. modified polymers, metal ions, polar solvents, and detergents), partition efficiency could be enhanced (Benavides *et al.*, 2008). For the aptamer-coated beads, the binding capacity was 120 μg of PDGF-BB/g of the beads. The beads are promising to be integrated with the other techniques to improve the purification efficiency. For instance, the aptamer-coated beads could be packed in a column used for chromatography, deposited onto the membrane, and/or suspended in the partition phase. Nevertheless the optimal conditions such as initial concentration of the protein, incubation time, temperature, require further investigations.

Moreover, binding interaction between the aptamers and their targets was compromised via hybridization between the aptamers and their CO (Soontornworajit *et al.*, 2010). Therefore, the hybridization could provide a way to elute the bonded proteins from the beads. PDGF-BB elution by CO has been investigated (Figure 5). The beads were treated with different CO concentration. Comparing to the initial amount of PDGF-BB added (250 pg), the amount of PDGF-BB from either non-CO treated sample or CO treated sample were approximately 16 pg, and they were not significantly different. Thus the elution process by CO was not successful even at high CO concentration. The aptamer might be shielded by PDGF-BB. Consequently, the CO did not have a chance to interact with the aptamer (Soontornworajit *et al.*, 2011). Optimization of CO sequence should be further investigated.

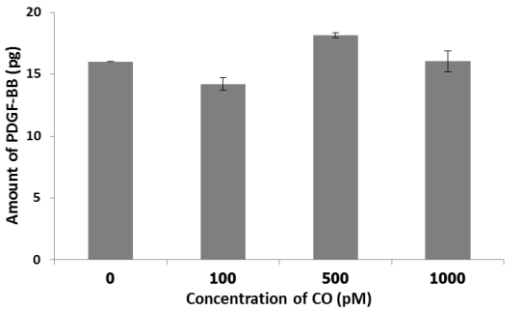


Figure 5 Effect of CO concentration on PDGF-BB elution.

4. Conclusions

Aptamer-coated beads for PDGF-BB capturing were developed by using EDC for amide coupling reaction. The concentration of EDC and PDGF-BB aptamer demonstrated a contribution on coupling efficiency. Meanwhile, the reaction was also preferable in acidic condition. The beads were able to capture the growth factor via a specific binding between PDGF-BB and its aptamer and a non-specific binding between this growth factor and the aptamer-coated beads. However the elution of PDGF-BB from the particles was not efficiently and required further study. The aptamer-coated beads are promising tools for using in a number

of biological and biomedical applications, especially a protein purification kit.

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