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Electrokinetic concentration on a microfluidic chip using polyelectrolytic gel plugs for small molecule immunoassay

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ABSTRACT

We have devised a new method to enhance the sensitivity of competitive immunoassay using electrokinetic concentration near a pair of highly charge-selective polymer [poly-2-acrylamido-2-methyl-1-propanesulfonic acid (pAMPSA)] plugs on a microfluidic chip. The polyelectrolytic gel, which was photopolymerized in a microfluidic channel network, served as the effective charge-selective extractor to sophisticatedly control the ion distribution. In this system, the fluorescent indicators on magnetic microbeads dispersed in the sample were spontaneously displaced by unlabeled target molecules and then electrokinetically preconcentrated in a single spot on the microfluidic chip. The locally preconcentrated fluorescent indicators were detected by laser-induced fluorescence. As a proof-of-concept, the competitive displacement assay of unlabeled 1 nM biotin was conducted to observe *ca.* 2000-fold enrichment within 3 min. In addition to the sensitive assessment of the unlabeled small target molecules, the proposed immunoassay system also showed good selectivity for biotin analogs such as biocytin, 2-iminobiotin, desthiobiotin.

1. Introduction

The characterization of trace amounts of biologically active small molecules (molecular weight less than 1000 Da) is critical in clinical and environmental analyses [1,2]. Many small molecules such as steoroid/thyroid hormones, catecholamine, eicosanoids, and synthetic drugs/herbicides act as important agonists or antagonists of specific target proteins [3]. As such, the small molecule–biomacromolecule interaction constitutes one of the most significant biological networks, encompassing protein–drug, protein–ligand, and enzyme–substrate complexes [4]. Conventional characterization methods for collection, identification, and/or quantitative analysis of small molecule–biomacromolecule interactions such as affinity chromatography [5], mass spectrometry [6], capillary electrophoresis [7], surface plasma resonance [8], nuclear magnetic resonance [9], and X-ray crystallography are available. However, most of these methods involve labor intensive, time-consuming, or complicated processes for sample preparation.

In general, immunoassay methods can be categorized into two, *i.e.* competitive and noncompetitive immunoassays. The competitive immunoassay has been mostly employed to analyze small molecules [10], which rarely have more than one distinct epitopes required for detection by noncompetitive immunoassays, representatively, sandwich-type assay. However, the sensitivity of the competitive immunoassays is much lower than sandwich-type immunoassay and severely relies on the affinity of antibodies [11]. In this regard, there have been many attempts to develop antibodies with higher antigen-binding affinities [12]. These efforts include genetic manipulation of antibodies and *in vitro* affinity maturation of antibodies [13]. Seeking new procedures is another strategy to improve the sensitivity of the competitive immunoassays that are applicable to small molecules. Recently a single-chain variable-fragment library was selected by the phage-display method for the discovery of a general or specific recognition module, and the analog which can differentiate the binding profile toward small molecule was rationally designed [14]. It requires no sophisticated

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antibody engineering to synthesize artificial antibodies with better affinity.

Preconcentration of the sample prior to analysis is a crucial step in microfluidic systems because it can directly lead to lower detection limit. There are many techniques available for sample preconcentration in microfluidic devices, including isotachophoresis [15–17], isoelectric focusing [18–21], filed-amplified sample stacking [22–24], solid-phase extraction [25–27], electrokinetic trapping [28–37], and temperature-gradient focusing [38]. Among such a variety of preconcentration schemes, electrokinetic trapping has received particular attention since the first demonstration at the interface between a micro- and nanochannel on a microfluidic chip [39]. There was another approach based on concentration polarization [40]. By creating an electric double-layer (EDL) overlap in charged nanochannels or nanoporous polymers, co-ions were excluded so that the nanostructure acted as a counterion permselective channel. When an electric field was applied across the ion-permselective nanochannel, local electric field gradients and the corresponding concentration polarization allowed sample preconcentration. With a simple fluid-handling protocol, this process enabled highly efficient analysis of any charged biomolecules on a microfluidic chip.

In our previous research, we brought polyelectrolytic gel electrode (PGE) into microfluidic chip systems for a variety of applications, *e.g.* velocimetry/cytometry [41], iontronic diodes/logic gates [42], complete blood cell counter [43], ultra-fast ion-depletion micromixer [44], and efficient preconcentrator [45,46]. The PGEs were photopolymerized in glass microfluidic channels and exhibited desirable properties for ion engineering including low impedance, high frequency response, good reproducibility and long-term stability. Highly charge-selective polymer [poly-2-acrylamido-2-methyl-1-propanesulfonic acid (pAMPSA)] for electrokineitc trapping possesses particularly useful characteristics for microfluidic preconcentration based on the phenomenon of concentration polarization [45]. In principle, the negatively charged polyelectrolytic gel (pAMPSA) plugs facing each other can be easily photopolymerized in the microchannel. Due to the anionic charge of the polyelectrolyte backbones, cations are selectively permitted to pass through the pAMPSA phases. When an electric field was applied across the two pAMPSA plugs, cations in the microchannel near the region between the pAMPSA plugs were extracted to the reservoirs in which the electrodes were immersed. Obviously, anions are rejected from the negatively charged polyelectrolytic gel plugs (pAMPSA) by electrostatic repulsion. If cations are extracted faster than supplied by mass transport in the microchannel, the local ionic charge balance breaks in the region between the pAMPSA plugs. As a consequence, an ion depletion region would appear and possibly expand as a function of the applied voltage and time [45]. Combined with electroosmotic flow (EOF), ion depletion could produce a spot at which ions were preconcentrated.

In this work, we propose a new way to enable even more sensitive detection of small molecules by combining the effective sample preconcentration on a microfluidic chip with bead-based competitive immunoassay. Biotin (cis-hexahydro-2-oxo-1-H-thieno-3,4-imidazolin-4-valeric acid; vitamin H) was selected in this study as a model of small targets because of its physiological importance as a water-soluble vitamin for cellular function and growth, thereby maintaining human health, vitality, and well-being. Fluorescent indicators (biotin-4-fluorescein) conjugated on magnetic microbeads were to be displaced by the unlabeled analytes. Subsequent electrokinetic preconcentration of the displaced indicators in the microfluidic chip markedly intensifies the signals from the preconcentrated indicators, leading to a novel strategy for sensitive immunoassay of unlabeled small analytes.

2. Experimental

2.1. Reagents

All reagents were used as received without further purification. Monoclonal mouse anti-biotin antibody, D-biotin, and biotin-4-fluorescein were purchased from Invitrogen (Carlsbad, CA, USA). Carboxyl-terminated magnetic microbeads (BioMag® Maxi) were received from Bangs Laboratories, Inc. (Fishers, IN, USA). Tris(hydroxymethyl)aminomethane was obtained from Acros (Geel, Belgium). 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPSA), 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (photoinitiator), *N*,*N*′-methlyenebisacrylamide (cross-linker), 3-(trimethoxysilyl)propylmethacrylate (TMSMA), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), bovine serum albumin (BSA), 2-iminobiotin, desthiobiotin, and sodium azide were purchased from Aldrich (St. Louis, MO, USA). Hexamethyldisilazane (HMDS) was received from J.T. Baker (Phillipsburg, NJ, USA). Sodium chloride, potassium chloride, sodium hydroxide, potassium phosphate dibasic, hydrochloric acid, and ethylenediaminetetraacetic acid (EDTA) were purchased from Daejung (Siheung, Korea). 18 MΩ cm deionized (DI) water was used to prepare all aqueous solutions (Barnstead NANOpure®, Thermo Scientific Inc., USA).

2.2. Preparation of antibody-conjugated magnetic microbeads

The conjugation of carboxyl-terminated magnetic microbeads with anti-biotin antibody was performed according to the instructions provided by the vendor (Bangs Laboratories, Inc.). Briefly, the magnetic beads (250 μL, 20 mg/mL) were washed twice in coupling buffer (10 mM K_2HPO_4, 0.15 M NaCl, pH 5.5) and magnetically separated. After addition of 100 μL EDC (40 mg/70 mL H_2O) solution, they were stirred for 15 min at room temperature. 250 μL of anti-biotin antibody (1.0 mg/mL) was added to the solution and stirred non-magnetically for 3 h at 37 °C. After conjugation, the mixture was magnetically separated to remove unbound antibodies. The antibody-conjugated magnetic microbeads were rinsed with washing buffer (10 mM Tris–HCl, 0.15 M NaCl, 0.1% w/v BSA, 0.1% NaN_3, 1 mM EDTA, pH 7.4), magnetically separated, redispersed in Tris buffer (10 mM Tris–HCl, pH 8.3), and finally stored in a refrigerator until used.

2.3. Competitive immunoassay

Different concentrations of biotin were used as target small molecule in this study. The target molecules (biotin) were diluted in Tris buffer (10 mM Tris–HCl, pH 8.3). A 250 μL volume of the antibody-conjugated magnetic microbeads was mixed with 250 μL of 10 μM fluorescent indicators (biotin-4-fluorescein) solution in a 1.5 mL Eppendorf tube, and resulting mixture was incubated for 1 h at 37 °C. After conjugation, the mixture was magnetically separated to remove unbound fluorescent indicators and resuspended in 250 μL of Tris buffer. And then, 250 μL of biotin solution was added to the suspension and incubated for 1 h at 37 °C for competitive immunoreaction. Subsequently, the solution of displaced indicators was retrieved by separating the magnetic microbeads under magnetic field and injected into a microfluidic chip for electrokinetic preconcentration.

2.4. Fabrication of microfluidic chips

Microfluidic chips with 60 μm wide and 30 μm deep microchannels were fabricated by standard photolithography as we reported previously [47]. In brief, Corning 2947 precleaned slide glasses