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Isocratic reporter-exclusion immunoassay using restricted-access adsorbents

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We introduce analyte-dependent exclusion of reporter reagents from restricted-access adsorbents as the basis of an isocratic reporter-exclusion immunoassay for viruses, proteins, and other analytes. Capto™ Core 700 and related resins possess a noninteracting size-selective outer layer surrounding a high-capacity nonspecific mixed-mode capture adsorbent core. In the absence of analyte, antibody-enzyme reporter conjugates can enter the adsorbent and be captured, and their signal is lost. In the presence of large or artificially-expanded analytes, reporter reagents bind to analyte species to form complexes large enough to be excluded from the adsorbent core, allowing their signal to be observed. This assay principle is demonstrated using M13 bacteriophage virus and human chorionic gonadotropin as model analytes. The simple isocratic detection approach described here allows a rapid implementation of immunoassay for detection of a wide range of analytes and uses inexpensive, generally-applicable, and stable column materials instead of costly analyte-specific immunoaffinity adsorbents.

1. Introduction

Chromatographic analysis of drugs and biomolecules has become increasingly common in the last three decades.[1–4] When combined with mass spectrometry, column chromatographic techniques serve as powerful analytical platforms with excellent resolution, sensitivity, and reproducibility. Complex clinical and bioprocess samples usually require very high selectivity and substantial sample preparation before chromatographic analysis,[5] however, posing obstacles to the wider use of standard modes of column chromatography. An alternative approach to the detection of analytes in complex samples is immunoassay, which uses antibodies coupled with highly-detectable reporters to achieve selectivity and sensitivity. One particularly robust form of immunoassay is the immuno-chromatographic lateral flow assay, best-known as the basis of the home pregnancy test. In our previous work, we improved the sensitivity of an immuno-chromatographic lateral flow assay for MS2 virus detection using functionalized viral nanoparticles as reporters.[6] This approach gave superior limits of detection but is not suitable for routine, automated, and quantitative analyses in a central clinical laboratory or process analytical technology setting.

To address these issues, we explored the replacement of the lateral-flow format with analyte-dependent exclusion of reporter reagents from restricted-access chromatographic adsorbents (Fig. 1a and b). Capto™ Core 700 is a restricted-access adsorbent composed of two layers: a highly cross-linked agarose core with multimodal capture ligands and a porous outer shell with a size-exclusion cut off of 700 kDa (Fig. 1a), combining the functionalities of size-exclusion and nonselective capture. The use of Capto Core 700 has been previously reported for process-scale purification of viruses.[7–13] In this study, we report the development of an isocratic immunodetection platform using Capto Core media to capture small reporters not bound to large or artificially-expanded analytes. Large

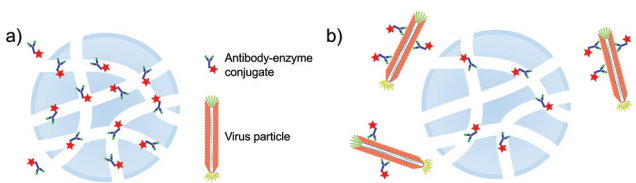

Fig. 1 Illustration of reporter-exclusion assay for large analytes such as viruses. (a) In the absence of large viral analyte, small protein reporters such as antibody-enzyme conjugates can penetrate the core of the resin particles and bind to the internal mixed-mode ligands. (b) In the presence of virus particles, antibody-enzyme conjugates bind to the viruses, are excluded by the size-selective shell, and can give detectable signal.

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analytes such as viruses can be detected directly by their ability to sequester small antibody-HRP conjugate reporters from capture in the adsorbent, and smaller analytes such as proteins can be detected by their ability to bridge reporter immunoconjugates onto carrier particles large enough to be excluded from the adsorbent.

2. Materials and methods

2.1 Cultivation of M13 phage

M13 phage was produced and titered as reported elsewhere.[6] Briefly, *E. coli* strain TG1 was grown to mid-log phase in Lysogeny broth (LB) medium. This pre-culture was then infected with M13 phage at 10^{12} M13 phage per mL, incubated at 37 °C for 2 h, then transferred to yeast extract tryptone (2xYT) medium and cultured overnight at 37 °C. After centrifugation, the sample was filtered through a 0.45 μm filter (cat. no. 430512, Corning®) and subjected to polyethylene glycol (PEG)/salt precipitation with 20% w/v MW 3350 g mol^{-1} PEG in 2.5 M NaCl.

2.2 Reporter-exclusion assay for M13 phage using restricted-access adsorbent

All experiments were carried out at 4 °C on an AKTA purifier 10 using Capto Core 700 (cat. no. 17548101, Cytiva, Uppsala, Sweden) media packed in a glass HR 5/5 column (36 mm bed height; 5 mm inner diameter; 0.7 mL column volume) and equilibrated with 1× phosphate-buffered saline (PBS) at a linear flow velocity of 122 cm h^{-1}. The bacteriophage M13 test analyte was incubated with (HRP)/Anti-M13 Monoclonal Conjugate (cat. no. 27-9421-01, Cytiva, Uppsala, Sweden) at a final reagent concentration of 2.5 μg mL^{-1}. This mixture was supplemented with 2.5 μL of culture supernatant of nuclease-producing *Serratia marcescens* per mL of sample volume to eliminate possible assay interference due to free phage nucleic acids.[14,15] After 1 h of incubation, the 500 μL sample was loaded onto the column, which was washed with 15 column volumes (CVs) of PBS buffer. One-mL fractions were collected and analyzed for HRP activity using 1-Step™ Ultra TMB-ELISA substrate (cat. no. 34028, Thermo Fisher™ Scientific).

2.3 Immunochromatographic protein assay using restricted-access adsorbent

To extend the method to smaller protein analytes as well as larger viruses, human chorionic gonadotropin (hCG) was chosen as a model analyte. Goat anti-α-hCG polyclonal antibody (pAb) was conjugated to M13 phage as described previously.[6] Briefly, 100 μL (10^{11}) M13 phage was suspended in 800 μL 3 mM EDTA in PBS. 2-iminothiolane (Traut's reagent, cat. no. 26101, Thermo Fisher Scientific) was added to a final concentration of 7 μM, and the reaction was incubated for 90 min at 25 °C. Traut's reagent reacts with the primary amines of M13 phage coat proteins to introduce reactive sulfhydryl groups. Excess Traut's reagent was removed using Zeba™ Spin Desalting Columns, 7 K MWCO (cat. no. 89882, Thermo Fisher Scientific). Maleimide activation of the goat anti-α-hCG pAb was performed by mixing the antibody and sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC, cat. no. 22322, Thermo Fisher Scientific) for 30 min at final concentrations of 22 μM and 1.14 mM, respectively, in 1 mL PBS. Sulfo-SMCC is a water-soluble heterobifunctional amine-to-sulfhydryl cross-linking reagent with a sulfo-NHS ester group and a maleimide reactive group linked by a cyclohexane spacer. Excess sulfo-SMCC was removed using Zeba Spin Desalting Columns, 7 K MWCO. Traut's reagent-modified M13 phage and sulfo-SMCC-modified pAb were then mixed for 90 min at 25 °C to allow coupling of the phage sulfhydryl groups to maleimide reactive groups on pAbs. Finally, uncoupled pAbs were removed using a 300 kDa Spectra/Por™ Float-A-Lyzer® (cat. no. G235036, Repligen™ Corporation). The M13 anti-hCG conjugate (10^8 mL^{-1}) was incubated with 10 ng mL^{-1} hCG in PBS for 30 min, followed by the addition of HRP/Anti-β hCG mAb conjugate (Thermo Fisher Scientific) to a final concentration of 2.8 μg mL^{-1}. This mixture was supplemented with 5 Units per mL of Benzonase Nuclease (cat. no. 70746, Millipore Sigma™). Commercial nuclease was chosen over the previous in-house nuclease to simplify sample prep and improve purity. The final mixture was incubated for 30 min at 25 °C. After incubation, 500 μL of the mixture was loaded onto the Capto Core 700 column. The column had been pre-equilibrated with PBS, and the assay was performed with PBS at a linear flow velocity of 31 cm h^{-1} (residence time 7 min). One-mL fractions were collected and analyzed for HRP activity using 1-Step™ Ultra TMB-ELISA substrate (cat. no. 34028, Thermo Fisher Scientific) and an ELISA plate reader (Tecan).

3. Results

3.1 Reporter capture by restricted-access adsorbent

To evaluate the feasibility of developing a reporter-exclusion virus immunodetection platform, we first tested the efficiency of capture of free HRP/Anti-M13 conjugate by the adsorbent. In the absence of M13 phage, a control sample containing HRP/Anti-M13 conjugate was largely trapped in the ligand active core of the Capto Core resin, although a residual HRP background signal at 450 nm (Fig. 2a, gray bars) and A280 (Fig. 2a, blue trace) in early fractions suggested incomplete capture of HRP/Anti-M13 conjugate and possibly other proteins from the nuclease preparation. In the presence of M13 phage analyte (Fig. 2b, gray bars), HRP/Anti-M13 conjugate bound to the phage was excluded from the adsorbent and detected in the eluted fractions. In volume fractions 4–6 (5–9 CVs), the HRP activity with analyte was at least 7 times that of the control without analyte.

To improve the completeness of reporter capture, these experiments were repeated at lower linear flow velocity (Fig. 2c), 31 cm h^{-1} for a residence time of 7 min *versus* the previous 1.8 min, with bed height kept constant at 36 mm. At a linear flow velocity of 31 cm h^{-1}, 99% of the HRP/Anti-M13

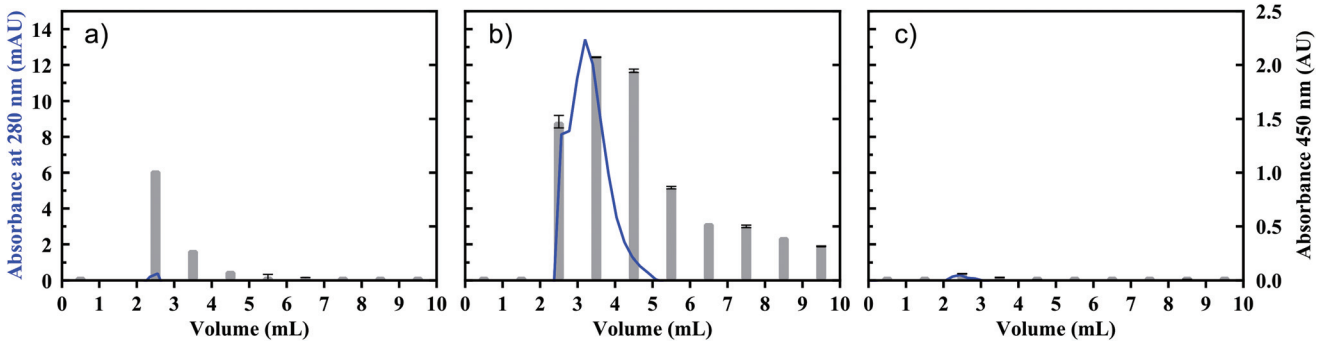

Fig. 2 Flow-rate dependence of reporter capture in a reporter-exclusion assay for M13 virus particles. (a) In absence of M13 phage, the HRP/Anti-M13 conjugate is almost entirely captured by the Capto Core 700 adsorbent at a high linear flow velocity of 122 cm h^{-1}; the uncaptured conjugate is detected as background HRP activity (gray bars). (b) At the same flow rate, for the sample containing M13 phage, the phage particles bind the conjugate and the HRP activity of excluded complex (gray bars) is detected and is much higher in later fractions than in the absence of phage. The blue trace indicates the absorbance of each fraction at 280 nm. (c) Loading HRP/Anti-M13 conjugate at a lower linear flow velocity of 31 cm h^{-1} (residence time 7 min) results in very low background HRP activity.

conjugate HRP activity was captured compared to HRP/Anti-M13 control sample (Fig. 2c, gray bars).

3.2 Isocratic detection of M13 phage using restricted-access adsorbent

The reporter-exclusion assay was performed with 10^7, 10^8, and 10^9 M13 phage particles per mL in PBS with a linear flow velocity of 31 cm h^{-1} (residence time 7 min). Fig. 3 shows that the assay can detect 10^7 M13 phage particles per mL, or less. In the absence of phage, the HRP/Anti-M13 conjugate was almost completely captured, resulting in low background HRP activity (<0.08 AU measured at 450 nm). The highest HRP activity, obtained at a volume of 3 mL (4 CVs), was 5-, 18- and 45-fold that of the no-phage control for samples containing 10^7, 10^8, and 10^9 M13 phage particles per mL, respectively.

3.3 Isocratic reporter-exclusion immunodetection of small protein analytes

The final size of the analyte-reporter complex is a critical aspect of a reporter-exclusion immunoassay using a restricted-access adsorbent. To enable the analysis of smaller analytes, we increased the size of the reporter-analyte complex by conjugating the capture antibody to M13 phage, here used as a passive size-increasing carrier particle (Fig. 4). Using a sandwich immunoassay format, the analyte is bound onto the large non-capturable phage particle which then binds the small antibody-reporter conjugate, producing a non-capturable carrier-analyte-reporter complex only when analyte is present. The assay was performed in PBS at a linear flow velocity of 31 cm h^{-1} (residence time 7 min), with column bed height 3.6 cm using 10^8 mL^{-1} M13 phage conjugated with anti-hCG

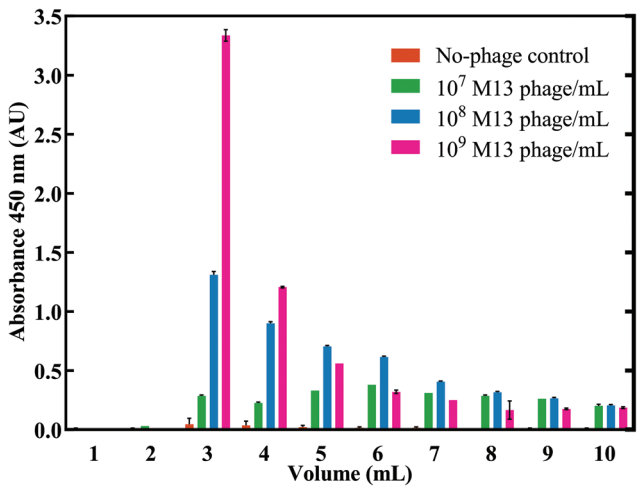

Fig. 3 Variation of reporter capture efficiency with phage concentration. Various concentrations of M13 phage incubated with HRP/Anti-M13 conjugate were separated at linear flow velocity 31 cm h^{-1} on the Capto Core column.

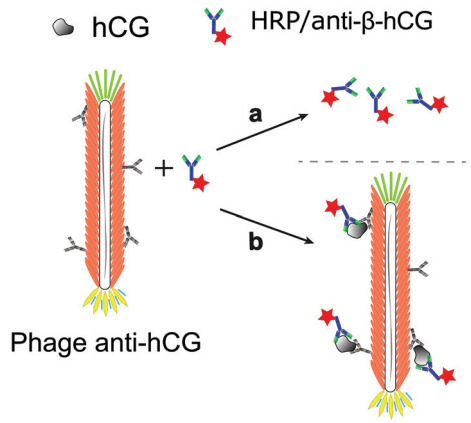

Fig. 4 Schematic of small-analyte reporter-exclusion immunoassay using restricted-access adsorbent. (a) In the absence of analyte, the free reporter is small enough to be captured by the adsorbent. (b) If present, analyte bridges the reporter onto phage, forming a large, excluded complex of anti-analyte M13 phage, sandwiched analyte, and anti-analyte reporter.

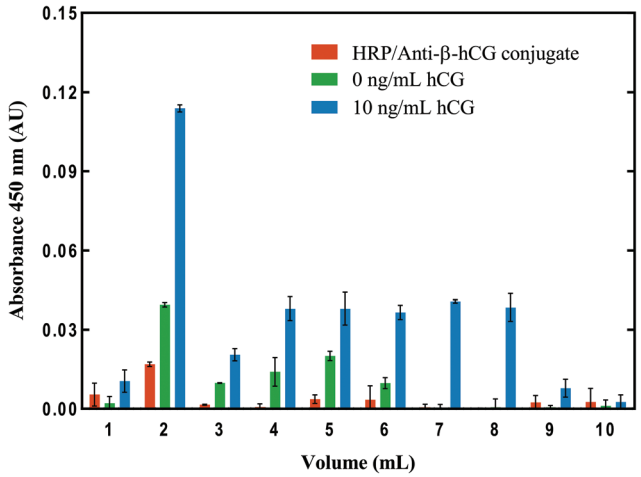

Fig. 5 Detection of hCG using M13 phage anti-hCG conjugate as an excluded capture carrier with HRP/Anti-β-hCG as reporter. Flow-through assays were performed with HRP conjugate alone, and with phage and conjugate without or with 10 ng mL^{-1} hCG. The HRP activity was analyzed using 1-Step™ Ultra TMB-ELISA substrate.

capture antibody and 4.34×10^{12} molecules of HRP/Anti-β-hCG reporter (1.4 μg) in a 500 μL load volume.

In the absence of hCG, this resulted in a low background signal (0.040 AU at 450 nm) as more than 99% of the HRP/Anti-β-hCG reporter was captured by the active core of the adsorbent (Fig. 5). In the presence of 10 ng mL^{-1} of hCG, the signal in fraction 2 (3 CVs) was at least 2.5-fold higher than the negative control without hCG, and this ratio was very high, up to 100-fold, in volume fractions 7 and 8.

4. Discussion

The application of chromatographic methods in immunoassays has been extensively explored over the last few decades. The most common technique, flow-injection analysis (FIA), has been used for immunoassay of many analytes.[16] FIA immunoassays usually are operated in either bind-elute or competitive displacement mode[16–18] and require costly, often single-use affinity adsorbents. The challenges of developing wash-free immuno- and ligand-binding assays have been addressed by several researchers; this work also has provided insights into the limitations of current approaches.[19–22] In previous work, we demonstrated a wash-free immunoassay based on the relocation of chemiluminescent reporters above a light-blocking dye by flotation on antibody-modified microbubbles.[23] The present work is conceptually related in transducing the presence of an analyte into the positioning of a reporter in a location where it is detectable.

In this work, we introduced the use of restricted-access adsorbent media as the basis for ligand-binding assays without the use of costly affinity columns. The Capto multimodal octylamine ligand is sufficiently nonspecific to capture the great majority of interfering molecules and reporters with varying charge and hydrophobicity.[24,25] The Capto Core media have been mainly studied for the purification of viruses or virus-like particles.[7,9–12,26] The use of multimodal ligands in chromatography is usually accompanied by an extensive pH and ionic strength screen[27–29] and use of mobile phase modifiers,[30,31] but these were not required for our immunoassay design. In this study, we first demonstrated detection of M13 phage particles using HRP/Anti-M13 reporters to show proof-of-concept. We then demonstrated the detection of a protein analyte to show the potential generality of this reporter-exclusion assay.

This platform can provide fast quantitation of viral titer at various stages of the vaccine manufacturing process. Specifically, it can be used to quantify the total virus content alongside the traditional TCID50 (Tissue Culture Infectious Dose) assay to obtain a ratio of infectious to non-infectious viruses, *e.g.*, in attenuated vaccines. The advantages of isocratic operation may also make the platform useful in veterinary, agricultural, food, and environmental applications. A theoretical limitation of our approach is saturation of the binding capacity of the Capto Core 700 adsorbent by high-protein samples such as blood or serum, but in practice, this is not expected to pose practical barriers. The samples of primary interest are urine, cell culture fluid, and serum with total protein concentrations of 6.2 mg per 100 mL,[32] 2.8 mg mL^{-1},[33] and 60–80 mg mL^{-1},[34] respectively. For the small column used here (700 μL), the dynamic binding capacity is 0.7 mL × 14.3 mg mL^{-1} (10 mg).[35] This capacity implies that 160 mL, 3.6 mL, and 120–170 μL of urine, cell culture fluid, and serum, respectively, can be loaded on this relatively small and inexpensive column; a larger one could easily be used. Adsorbents with greater selectivity, such as antibody affinity matrices, also could overcome this difficulty, extending the advantages of this assay format to a wider variety of applications though at greater cost.

The readout time required for the colorimetric assay can be avoided by changing the enzymatic colorimetric reporters to either fluorescence or luminescence reporters, which might also allow simultaneous detection of distinct analytes by selecting reporter materials with different emission wavelengths. The M13 phage could be replaced in small-analyte assays with any other suitable nanoparticle small enough to traverse the column and large enough to be excluded from the capture resin. In the present work, we have utilized a pump-driven liquid chromatography system for flexibility during initial assay demonstration, but more portable assays driven by gravity or a manual syringe could be developed along similar lines.

Conclusions

In this work, we introduce an assay format in which the presence of analyte is transduced into reporters spared from capture by restricted-access adsorbents. In addition to supporting isocratic immunoassays, this approach can be generalized

to a broad range of ligand-binding assays, using many types of reporters and recognition agents.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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