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Study on molecularly imprinted nanoparticle modified microplates for pseudo-ELISA assays

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ABSTRACT

Nanosized Molecularly Imprinted Polymers (nanoMIPs) are designed artificial nanoreceptors with a predetermined selectivity and specificity for a given analyte, lately proposed as a replacement to antibodies in immunoassays. The nanoMIP-plate preparation based on nanoparticle adsorption was studied with the aim to rationally identify and discuss the critical points in the nanoMIP-assay development, in an example based on the iron homeostasis biomarker hepcidin and hepcidin-specific nanoMIPs (K_d = 9 nM). Plates were prepared by deposition and drying of nanoMIP (0.5–4 µg/well), or by nanoMIPs co-depositions (proteins, PVA). Rehydration (> 1 h) of dry nanoMIP-plates showed the reconstitution of the imprinted binding sites. NanoMIP-plate mechanical stresses (several washings; pipetting) caused nanoMIP desorption (~90%). After 10 washes the quantity of nanoMIP was 0.2 µg/well, the imprinted binding sites were ~270 fmol/well, their accessibility the 92%. Co-depositions resulted in higher amount of adsorbed nanomaterial (1.2 µg/well), but low accessibility of the imprinted binding sites (2–47%).

Tested in a competitive sequential assay, using as competitor horseradish peroxidase conjugate to hepcidin, the nanoMIP-plate permitted to determine hepcidin in serum samples, yet with a narrow dynamic range of response (0.9–10 nM). Critical points in the assay were: the instability of the nanoMIP adsorption, which lead to the progressive loss of binding sites/well, and the affinity of the nanoMIP for the analyte (K_d = 9 nM), which corresponds to kinetics dissociation constants on the time-scale of the washing lengths (minutes), thus compatible with the release of the bound hepcidin during the washings. The found limits set the conditions to develop a successful nanoMIP-assay: (i) stable microplate derivatization; (ii) maximized number of imprinted binding sites/well; (iii) nanoMIP/analyte equilibrium not perturbed on the time scale of the minutes (i.e. K_d ~ pM).

1. Introduction

Molecularly imprinted polymers (MIPs) are a class of synthetic receptors prepared by a template assisted synthesis [1,2]: monomers are polymerized in the presence of the analyte of interest, called the template, so that impressions are left onto the growing polymer, ultimately conferring to it geometrical and chemical complementarity towards the template, in resemblance to biological receptors. When compared to their natural counterparts, MIPs exhibit an increased stability to harsh conditions, low costs of production, ease of integration to electronics and tailor made selectivity, as demonstrated by the successful imprinting of small molecules [3], proteins [4], large macromolecular assemblies [4]. Given these characteristics, the use of MIPs as a replacement of the antibodies in immunoassays [5–7] was explored since the pioneering works in the field [8–11]. The integration of MIPs to the immunoassay was realized by direct polymerization of a MIP layer at the bottom of the microplate well [10–15], in examples of imprinted conjugated aromatic polymeric films, such as aminophenylboronic acid, thiophenes, anilines that assured strong adhesion to the polystyrene microplate through π-π stacking [12–15]. Recently advancements in the MIP synthesis, such as precipitation polymerization [16], post-dilution [17], solid phase synthesis [18], directional syntheses [19,20] allowed to scale down the material to the nanometric dimension (20–200 nm), hitherto called nanoMIPs [21–23]. The nanoMIPs showed closer resemblance to the antibodies: low number of binding sites per nanoparticle [22,23], fast kinetics of interaction and high affinities, while sharing the robustness and shelf-lives of polymers, hence the nanoMIPs were sought as ideal “plastic antibodies” to be integrated in assays [24]. The concept was proven in a nanoMIP-based ELISA-assay for the detection of vancomycin in plasma [25], followed by nanoMIP-assays for few other analytes, including proteins [26]. Conversely an ELISA-mimic system based on immobilized protein targets (e.g. histone,

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fibrinogen) was used to quickly screen soluble polymeric nanoparticles, including nanoMIPs,for their affinity and selectivity towards the target [27]. Here we studied the process of nanoMIP-plates preparation, with particular attention to the nanoMIP adsorption and to the implications to the sensitivity and dynamic range of the assay, in an example based on the clinical biomarker hepcidin, key regulator of iron homeostasis [28,29]. In a previous study we developed aqueous compatible, high affinity and selective nanoMIPs for hepcidin [23]. Here the same nanoMIPs were used to prepare a pseudoELISA nanoMIP-assay, with the aim to identify and discuss the critical points in the methodology development.

2. Materials and methods

2.1. Materials

Acrylamide (Aam), Methacrylic acid (MAA), N-*t*-butylacrylamide (TBAm), N,N′-methylenebisacrylamide (BIS), N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium persulfate (APS), sodium dodecyl sulfate (SDS), sodium dihydrogen phosphate, sodium monohydrogen phosphate, sodium chloride, hydrochloric acid, Trizma® base, 2-[N-morpholino]ethanesulphonic acid (MES), N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (NHS), sodium acetate, Tween-20, ethanol, bovine serum albumin (BSA), k-casein, horseradish peroxidase (HRP), and 3,3',5,5'-tetramethylbenzidine (TMB) were from Sigma-Aldrich (Darmstadt, Germany). Polyvinylalcohol (PVA) Mn 35,000 Da and Acryloxyethyl thiocarbamoyl Rhodamine B were from Polysciences Inc. (Warrington, USA). BRANDplates® lipoGrade™ and BRANDplates® hydroGrade™ were from BRAND GMBH CO KG (Wertheim, Germany). Synthetic hepcidin-25 was from Peptide International (Louisville, KY, USA). Serum samples were from healthy volunteer donors. The template peptide of sequence DTHFPI was custom made by TAG (Copenhagen, Denmark). Washing buffers: 20 mM sodium phosphate pH 7.2, 154 mM NaCl 0.05% Tween-20 (PBS-T); 20 mM PBS 0.5% Tween-20 (PBS-05T); 20 mM PB with 0.5% Tween-20 (PB-05T).

2.2. Synthesis of the nanoparticles

The synthesis of the imprinted nanoparticles (nanoMIP) and of the control non-imprinted nanoparticles (nanoNIP) were according to the protocol of Cenci et al. [23]. Fluorescent nanoparticles (nanoRs) were prepared using the same composition of nanoNIPs but with the monomer acryloxyethyl thiocarbamoyl Rhodamine B (2% mol:mol) (SM).

2.3. Dynamic light scattering

Size distribution and polydispersity index (PDI) of the nanoparticles were determined by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZEN3600 (Malvern Instruments Ltd, Worcestershire, UK) equipped with a 633 nm He–Ne laser. Nanoparticles were dissolved in PBS-T to a final concentration of 1 mg/ml and filtered 0.22 µm. A nanoparticle refractive index (RI) of 1.590 and an absorption value of 0.01 were assumed. Detection was at an angle of 173°. Samples were measured in triplicate. Rehydration was followed over a time course of 2 h after resuspension.

2.4. NanoMIP/ligands interaction studied by isothermal titration calorimetry (ITC)

A Nano ITC Standard Volume (TA Instruments, Newcastle, US) with a fixed gold cell was used. NanoMIPs were suspended at 1.2 µM in 50 mM PBS-T, sonicated and let to rehydrate for 2 h. Hepcidin, the template peptide DTHFPI, HRP, and the tracer were prepared at 4 or 10 µM in PBS-T. All samples were degassed under vacuum for 15 min prior to the analysis. The reference cell was filled with 200 µl of degassed water, the sample cell was filled with 200 µl of nanoMIPs, 50 µl of titrant solution was loaded in the syringe. ITC experiments consisted of 12 injections of 4 µl, interval 300 s, run in triplicate, temperature was 25 °C, stirring speed 250 rpm. The dissociation constant (Kd), enthalpy (ΔH°), entropy (ΔS°) and free energy variation (ΔG°) were calculated by fitting data with the independent site model (Nano Analyze Software v. 3.4.0, TA Instruments, New Castle, DE).

2.5. Atomic force microscopy

The Atomic Force Microscopy (AFM) images were acquired using SMENA system from NT-MDT, equipped with NSG10 probes. A drop of nanoMIPs (5 µg/ml in water) was deposited on a clean silicon wafer and let dry. Images at 5 × 5 μm^2 and 1 × 1 μm^2 were acquired in tapping mode and analyzed with the Gwyddion software [30].

2.6. Deposition of nanoparticles to the microplate

NanoR, nanoMIP and nanoNIP, re-suspended in water, were deposited at 0.5, 2 or 4 µg/well onto either the lipophilic microplates (BRANDplates® lipoGradeTM, BRAND GMBH + CO KG, Wertheim, Germany) and the hydrophilic microplates (BRANDplates® hydroGradeTM, BRAND GMBH + CO KG, Wertheim, Germany), to reproduce the conditions of [25,26]. Microplates were dried O/N at 28 °C. For the co-depositions, the nanoparticles (4 µg/well) were mixed to: 0.2 mg/ml of BSA; 0.2 mg/ml casein; 0.2% (w/v) PVA Mn 35000 g/mol, let O/N at 28 °C. The quantity of adsorbed nanoparticles was measured by fluorescence emission at λ = 590 nm. A calibration curve was built by measuring the fluorescence emission of known nanoR quantities at 590 nm (with an excitation wavelength of 550 nm) on a VICTOR[3] 1420 Multilabel Counter (PerkinElmer™, Waltham, Massachusetts, USA). The equation of the curve was: y (RFU) = × 29.92 (µg) – 2.41; R^2 = 0.998.

2.7. Stability of the nanoMIP-plate

Nanoparticle deposited and co-deposited microplates were washed 10 times with 200 µl/well of PBS-T PBS-05T or PB-05T by shaking the microplate at 100 rpm for 2 min. The quantity of adsorbed nanoR was monitored by measuring the fluorescence emission as described above. The experiments were repeated onto 3 plates and for 2 batches of nanoparticles.

2.8. Preparation of the tracer

According to [31] three equivalents of the N-terminus peptide of hepcidin (the hexapeptide of sequence DTHFPI) were dissolved in 10 mM MES pH 5 and activated for 15 min with EDC/NHS 0.01/0.025 M respectively. One equivalent of HRP was added after the pH was adjusted to 6.2 and the mixture was incubated for 2 h at room temperature. The tracer DTHFPI-HRP was purified on a FPLC ÄKTA (GE Healthcare, Little Chalfont, UK) pure equipped with a size exclusion chromatography Superdex G200 column (GE Healthcare, Little Chalfont, UK), quantified by measuring the absorbance at 403 nm and stored at −20 °C.

2.9. Binding capacity of the nanoparticles

NanoMIPs/NIPs were dissolved in PBS-T. Four µg of nanoparticles were incubated with increasing concentrations of the tracer from 0.02 to 15 nM for 1 h at room temperature. The samples were washed thrice with 200 µl of PBS-05T on a 100 K Amicon® Ultra Centrifugal Filters (Merck Millipore Ltd., Billerica, Massachusetts, USA) then, 50 µl of each sample were transferred into a microplate and 50 µl of TMB were added. After 15 min of incubation in the dark, the reaction was stopped

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