Journal of Colloid and Interface Science 348 (2010) 1–8

Contents lists available at ScienceDirect

Journal of Colloid and Interface Science

www.elsevier.com/locate/jcis

Improved functionality of antibody-colloidal gold conjugates with the aid of lipoamide-grafted *N*-[tris(hydroxymethyl)methyl]acrylamide polymers

Willem M. Albers [a,*], Tony Munter [a], Päivi Laaksonen [b], Inger Vikholm-Lundin [b]

[a] VTT Technical Research Centre of Finland, Molecular Sensors, P.O. Box 1300, FI-33101 Tampere, Finland
[b] VTT Technical Research Centre of Finland, Nanobiomaterials, P.O. Box 1000, FI-02044 Espoo, Finland

ARTICLE INFO

Article history:
Received 22 February 2010
Accepted 26 March 2010
Available online 1 April 2010

Keywords:
Colloidal gold
Antibody Fab′-fragment
Immobilisation
Stability
Surface Plasmon Resonance
Sandwich immunoassay

ABSTRACT

Colloidal gold has been used as a label in sandwich assays for human IgG, in which intercalating *N*-[tris(hydroxymethyl)methyl]acrylamide (pTHMMAA) polymers have been employed to stabilise the particles coated with antibody fragments. A direct absorbance reading of the particles could be obtained from sandwich assays on polystyrene, and a strongly amplified response was observed in similar assays based on Surface Plasmon Resonance (SPR): for h-IgG, detection limits below 100 pg/mL could be achieved. Three different polymer lengths and two different particles sizes were compared in sandwich assays performed on polystyrene and gold. The resulting binding curves fitted well to the Langmuir–Freundlich isotherm and the binding constants were in good agreement with the values found in earlier studies. The amplification afforded by the nanoparticles was strongly dependent on the antigen concentration, on the type of polymer and on the particle size. Compared to the direct response of the antigen, amplification factors larger than 100 could be achieved. The study proves that the polymers give stabilised particles, which can be used in highly sensitive sandwich assays.

1. Introduction

Surface Plasmon Resonance (SPR) has become an established detection method for the study of biomolecular interactions in real-time, as evidenced by a strong growth in the number of commercially available SPR instruments [1,2]. In the scientific arena, development of SPR instrumentation has presently moved into a stage of microfabrication and multi-analyte detection, mainly to alleviate cost of the sensing system for future point-of-care (POC) devices [3,4]. Although molecules with a low molecular weight can be determined fairly well with state-of-the-art biosensing instruments in the higher nanomolar range, the reference ranges of many analytes of clinical interest lie still far below the range that is assessable with direct SPR detection. This is mainly due to the slow reaction kinetics at concentrations below nanomolar, even when mass transport-limitations are removed [5]. Consequently, a great variety of studies have appeared in which sensitivity and selectivity of SPR detection have been enhanced with gold or latex nanoparticles [6]. With gold nanoparticles (AuNPs), the SPR sensitivity is considerably enhanced not only due to the high refractive index effect but also due to the electronic coupling between the localized surface plasmons of the AuNPs and the plasmons at the surface [7,8]. Proteins and DNA are mostly adsorbed onto the particle surface via weak physical interactions ('physisorption') [9,10]. Both competitive and sandwich assay formats have been reported that display very high detection sensitivity [11–16]. For instance, Besselink et al. have made a comprehensive study on various SPR detection techniques for human prostate-specific antigen (PSA), including a comparison of hydrogel-modified gold surfaces and planar surfaces for capture antibody immobilisation, using both latex and gold particles as a label for enhancement of the SPR signal [14]. The results were also backed up with theoretical calculations. The highest sensitivity for PSA detection could be obtained on planar surfaces, attaining a lower limit of detection of 0.6 and 2.4 ng/mL respectively for gold and latex particles. Recently, Choi et al. have claimed a detection limit for PSA of 10 pg/mL with a sandwich assay, in which protein G was used to immobilise the monoclonal capture antibody on the sensor surface, while a polyclonal antibody conjugated to AuNPs was used for detection [13].

We have recently investigated polyacrylamide polymers with tris(hydroxymethyl)-functional groups (pTHMMAA) to produce highly functional and stable layers of antibodies on gold, in which the polymer molecules were co-assembled with antibody Fab′-fragments. It was demonstrated that the polymer pTHMMAA intercalates under various conditions between the antibody molecules and improves immunoassay characteristics by suppressing non-specific binding and increasing the orientation of the antibody molecules [17–21]. This is likely due to the bulkiness and the

* Corresponding author. Fax: +358 20 722 3319.
E-mail address: martin.albers@vtt.fi (W.M. Albers).

0021-9797/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jcis.2010.03.059

highly hydrophilic nature of the TRIS head group, which limits the intra-molecular interactions between the protein and the polymer to hydrogen bonding interactions. Arguably, the hydrogen bonding with biomolecules is reduced by a high degree of hydrogen bonding with water and within the polymer itself [22]. Thus, these polymers can be considered as an interesting alternative for other types of non-fouling polymers based on zwitterionic phosphatidyl choline or polyethylene glycol moieties [23], or on hyperbranched polyglycerols [24]. We have earlier shown that similar polyacrylamide polymers are able to form highly stabilised gold colloids already at low concentration [25]. As a logical consequence, we embarked on the preparation of intercalated layers of antibody Fab′-fragments and pTHMMAA on gold nanoparticles, of which preliminary results are presented in this paper. We demonstrate that the pTHMMAA polymer has a beneficial effect on the stability of the gold nanoparticles and that amplification factors above 120 could be obtained with Fab́-fragments and pTHMMAA coated onto the AuNPs.

2. Experimental

2.1. Materials and instrumentation used

2.1.1. Chemicals

4,4-Azobis(4-cyanopentanoic acid)-*N,N*-disuccinimidyl ester and *N*-(3-aminopropyl)–lipoamide were prepared according to literature procedures [26,27]. The solvents (analytical grade) were purchased from Merck KGaA. MilliQ water was used with a resistance larger than 18 M and a TOC of 4 ppb or lower.

2.1.2. Immunoreagents

Affinity-purified polyclonal goat anti-human IgG and corresponding $F(ab')_2$-fragments against either the Fc_γ-domain or the $F(ab')_2$-domains of human IgG were obtained from Jackson Immunoresearch Labs Inc. (PA, USA). Antibodies against the Fc_γ-domain were generally employed as capture antibodies on polystyrene and gold surfaces, while the antibodies specific for the $F(ab')_2$-domains were immobilised onto the gold colloids. The antigen, human IgG (h-IgG), was a chromatographically purified preparation also from Jackson Immunoresearch Labs. Bovine serum albumin, with a purity >98%, and dithiotreitol were obtained from Sigma–Aldrich (Rochester, USA). Centrifugal dialysis devices with a cut-off of 3 kDa ('Nanosep 3K OMEGA') were obtained from Pall Corporation (Ann Arbor, Michigan, USA). Phosphate buffered saline (PBS) consisted of 150 mM sodium chloride and 10 mM sodium phosphate at pH = 7.4 and PBS/EDTA buffer contained 150 mM sodium chloride, 5 mM EDTA and 50 mM sodium phosphate, pH = 7.4. Assay buffer contained 0.5 mg/mL BSA, and PBS/Tween contained 0.05% Tween 20, both dissolved in PBS buffer. Microtitre plates (of type "Maxisorb"), were obtained from Nunc A/S (Roskilde, Denmark). Gold substrates for SPR were prepared in-house by RF magnetron sputtering, using an Edwards E306A two-target sputter coater [17]. A microplate reader, type Spectra Rainbow (Tecan AG, Switzerland) was used for flocculation experiments and recording of dose–response curves on polystyrene. SPR measurements were performed on a Biacore 3000 instrument (GE Healthcare/Biacore AB, Uppsala, Sweden).

2.1.3. NMR and MS

The ^1H and ^{13}C NMR spectra were recorded with a 300 MHz Varian NMR spectrometer operating at frequencies given with the spectral data. The solvent was used as the internal reference standard. The mass spectrometric analyses were performed on an Agilent 1100 Series LC/MSD Trap system (Agilent Technologies, Espoo, Finland) equipped with an electrospray source and operated in the positive ion mode.

2.1.4. Chromatography and dialysis

Thin layer chromatography (TLC) was performed on silica gel 60 F_{254} TLC aluminium sheets (Merck KGaA). The spots were visualised by dipping the TLC plate in an alkaline aqueous solution of potassium permanganate or a solution of ninhydrin in 1-butanol/acetic acid (100/3 vol/vol). For column chromatography, silica gel 60 with a particle size of 0.04–0.063 mm (230–400 mesh) (Fluka, Sigma Aldrich Finland Oy (Helsinki, Finland) was used. HPLC analyses were performed on a Shimadzu Prominence HPLC system consisting of two LC-20AD pumps, a SPD-M20A diode-array detector and a DGU-$20A_3$ degasser. The polymer solutions were chromatographed on a 8 × 300 mm OHpak SB-802.5 size exclusion column (SHODEX). The eluent consisted of 10% methanol in an aqueous solution of 50 mM NaCl. The flow rate was 0.5 mL/min. The polymers were purified by dialysis against water in a Spectra/Por *'float-a-lyzer'* tube (10 mL) with a cellulose ester membrane (molecular weight cut-off 5000) (Spectrum Laboratories Inc.).

2.1.5. TEM-measurements

Size distributions of the nanoparticles were measured with transmission electron microscopy (TEM) using a Tecnai 12 instrument operating at a 120 kV accelerating voltage. The samples were prepared by placing a drop of nanoparticles dispersed in water on holey carbon grids (Agar Scientific). The grids were let to dry in air before measurement. Size analysis of the particles was carried out using ImageJ software (public domain software, National Institutes of Health, USA).

2.2. Preparation of azo-initiator **3**

To a solution of *N*-(3-aminopropyl)–lipoamide (**2**) (0.66 g, 2.5 mmol) in methanol (80 mL) was added *N,N*-dimethylformamide (DMF) (15 mL) and the methanol was removed by rotary evaporation. The solution of the compound in DMF was added to a suspension of 4,4-azobis(4-cyanopentanoic acid)-*N,N*-disuccinimidyl ester (**1**) (0.569 g, 1.2 mmol) in DMF (30 mL). The reaction mixture was stirred at room temperature, while the disappearance of **2** was monitored by TLC [methanol/ammonium hydroxide (28–30% NH_3), 100/7 vol/vol, ninhydrin-dip]. After 23 h, dichloromethane (170 mL) was added and the mixture was washed with brine (2 × 80 mL) and water/brine (100/10 vol/vol) (5 × 100 mL). The dichloromethane phase was dried (Na_2SO_4), filtered and chromatographed on silica gel [eluent dichloromethane/methanol (100/8 vol/vol), TLC $KMnO_4$-dip]. The solvent was removed by evaporation under reduced pressure and the residue was dried under vacuum to give the azo-initiator (**3**) as a light-yellow powder (0.6 g, 65%). ^1H NMR (300 MHz, DMSO-d_6): δ 7.95 (*t*, 2H, *J* = 5.5 Hz, 2 × N*H*), 7.76 (*t*, 2H, *J* = 5.5 Hz, 2 × N*H*), 3.60 (*m*, 2H, 2 × C*H*SS), 3.13 (*m*, 4H, 2 × CH_2SS), 3.03 (*m*, 8H, 4 × CH_2NH), 2.47–2.12 (*m*, 10H, 2 × $CH_a$$CH_2$SS + $CH_2$$CH_2$CCN), 2.05 (*t*, 4H, *J* = 7.2 Hz, 2 × CH_2CONH), 1.86 (*m*, 2H, 2 × $CH_b$$CH_2$SS), 1.69 (6H, 2 × CH_3), 1.66–1.21 (16H, 2 × SSCH$CH_2$$CH_2$$CH_2$ + 2 × $CH_2$$CH_2$$CH_2$NH). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 171.9, 169.8, 118.2, 72.0, 56.1, 39.9, 38.1, 36.5, 36.3, 35.2, 34.1, 33.2, 30.0, 29.2, 28.3, 25.1, 23.3. ESI-MS: *m/z* 769 (100, MH^+).

2.3. Preparation of the polymers

The polymer **4** was essentially prepared according to earlier published methods [17,20,28]: to *N*-[tris-(hydroxymethyl)methyl]-acrylamide (317 mg, 1.8 mmol) in 10 mL of ethanol/water (4/1 vol/vol) was added a solution of azo-initiator **3** (28 mg, 0.036 mmol) in DMF (5 mL). The mixture was degassed in vacuo by repeated