A double-bead sandwich assay for protein detection in serum based on Localized Surface Plasmon Resonance

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ABSTRACT

In this study, the importance of pre-concentration is clearly demonstrated for the covalent immobilization of various proteins to magnetic nanoparticles through mild carbodiimide activation. Additionally, the covalently biofunctionalized magnetic nanoparticles are employed in a double-bead sandwich assay, using gold nanoparticles. The magnetic nanoparticles allow specific capturing of the analyte in biological samples (serum), while the optical properties of the gold nanoparticles provide the signal transduction. We demonstrated that a major improvement in the assay sensitivity was obtained by selecting an optimal gold nanoparticle size (60 nm). A detection limit of 5-8 ng/mL, a sensitivity of 0.6-0.8 $(pg/mL)^{-1}$ and a dynamic range of 3 orders of magnitude were achieved without any further amplification using the detection of prostate specific antigen in serum as a model system. The proposed assay has the ability to be easily implemented within a microfluidic device for point-of-care applications whereby the readout can be executed by a fast and cheap optical measurement.

Keywords: immuno-assay, gold nanoparticles, magnetic nanoparticles, localized surface plasmon resonance

1 INTRODUCTION

Biosensor research is evolving towards nanoparticle based biosensing. The physical and biological properties of biofunctionalized nanoparticles have attracted many researchers and inspired them towards the development of ultra sensitive biosensor devices. For the biofunctionalization of suspended nanoparticles various strategies have already been explored. Beside adsorption, chemisorption and covalent immobilization of proteins onto the nanoparticle surface, the covalent bond is envisaged to have the most potential. An important aspect in the covalent biofunctionalization of nanoparticles has been neglected, namely pre-concentration. Exploiting the electrostatic attraction forces between a protein and the nanoparticle surface will favor the covalent immobilization.

The integration of nanoparticles into lab-on-a-chip devices has the potential to improve point-of-care applications. For example, nanoparticles have been used for the detection of analytes and for a combined detection and controlled transport of cells, proteins and DNA. These, so-called 'bead-based immunoassays' offer several advantages over traditional, two-dimensional technologies. Most importantly, nanoparticles bare a large surface area and the reactions are no longer governed by planar diffusion, but by a faster radial diffusion. In this presentation, a simple, general, low cost, and easy to automate double bead-based immunoassay has been developed using commercially available MNPs and GNPs. By introducing a proper biofunctionality, the magnetic properties of the MNPs allow easy and selective isolation of the analyte, even in complex matrices such as serum. On the other hand, biofunctionalized GNPs can selectively bind the analyte isolated by the MNPs. Moreover, the bright extinction of GNPs makes them ideal candidates for signal transduction.

In this study, the detection of prostate specific antigen (PSA), which is a marker for prostate cancer, was chosen to demonstrate the effectiveness of the proposed double-bead assay in both buffer and serum environment. Normally, prostate cancer is suspected if the total PSA level is higher than 10 ng/mL. Sensitive and specific detection of PSA (sub ng/mL) for early prostate cancer detection is of great importance, even towards point-of-care applications.

2 MATERIALS AND METHODS

2.1 Materials

GNPs of different sizes (40, 50, 60, 80 and 100 nm) were purchased from BBInternational (Cardiff, UK). 20 nm GNPs were synthesized as described in our previous research. [1] Acetic acid, boric acid, 4-(2-hydroxyl-ethyl)-1-piperazineethanesulfonic acid (HEPES), Tween20®, bovine serum albumin (BSA) and all other chemicals were obtained from Sigma-Aldrich (Missouri, USA). Monoclonal IgG type antibodies for the capturing of prostate specific antigen (PSA) in a sandwich approach, i.e. PSA66 (310) and PSA19 (301), were obtained from Fujirobio (Pennsylvania, USA). PSA and normal female serum samples were obtained from Scipac (Kent, UK). Carboxyl functionalized magnetic nanoparticles (MNPs) with an average diameter of 300 nm were purchased from Ademtech (carboxyl-adembeads, Pessac, France). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-

hydroxysuccinimide (NHS) were purchased from BIAcore (GE Healthcare, UK). The used HBS buffer contains 10 mM HEPES, 150 mM NaCl, 0.005% (v/v) Tween20® and 3.4 mM ethylenediaminetetraacetic acid (EDTA). The pH was adjusted to 7.4 using sodium hydroxide. All chemicals and biomolecules were used as received.

2.2 Methods

The detailed information concerning the covalent biofunctionalization of the MNPs can be found in [2]. The adsorption of biomolecules onto the GNPs was performed based on our previous research and thoroughly characterized as described in [3]. Additionally, all the details concerning the conduction of the sandwich immunoassay are discussed in [4]. The absorbance measurements were performed using a Shimadzu UV-2550 spectrophotometer (Japan). Zeta-potential and size measurements were performed using the Zetasizer nano ZS system of Malvern Instruments (UK) and the SEM images were taken using a Philips XL30 FEG instrument operating at an acceleration voltage of 5 kV. The extinction cross-section of the GNPs was calculated using MiePlot v.3.4.12 (Philip Laven, Switzerland). All calculations were performed for spherical GNPs in water (5°C, density: 0.99996382 kg/m3).

3 RESULTS AND DISCUSSION

3.1 Impact of pre-concentration to covalent biofunctionalization of MNPs.

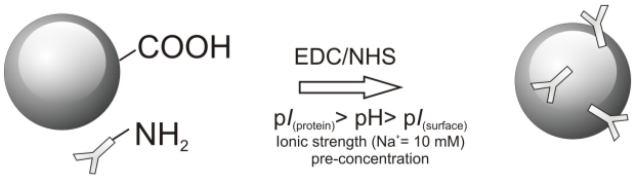

Figure 1: Schematic representation of the covalent biofunctionalization strategy.

Carboxyl functionalized magnetic nanoparticles (MNPs), i.e. carboxyl-adembeads, were chosen to covalently biofunctionalize with antibodies. As a cross-linker, a combination of EDC and NHS was used to bind the antibodies via their lysine residues (-NH_2) as presented in figure 1. The reaction conditions were optimized such that nanoparticle aggregation is avoided (11 mM EDC/NHS). The antibodies of interest were dissolved in a suitable buffer solution with low ionic strength and ideal pH to favor the covalent immobilization. The latter parameters were depicted from the strategy used to immobilize proteins onto a carboxymethyldextran modified gold surface for biospecific interaction analysis in a Surface Plasmon Biosensor, i.e. BIAcore [5]. Maximizing the electrostatic attractive force between the antibody and the carboxyl surface leads to an accumulation of the proteins close to the surface, i.e. pre-concentration.

To determine the optimal conditions for the antibody immobilization onto MNPs, an indirect approach was used whereby the biofunctionalized MNPs were incubated with their target analyt labeled with GNPs. In this way, the amount of GNPs correlates with the amount of immobilized antibodies onto the MNP surface. The antibody coupling efficiency, applying different pre-concentration buffers, is shown in figure 2. The optimal pre-concentration buffers for the biofunctionalization of the MNPs with respectively antibody 310 and 301 are acetate pH 5.5 and pH 4.5.

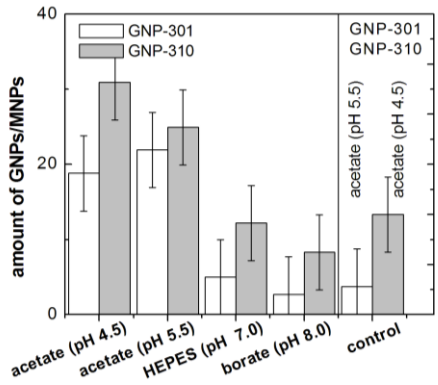

Figure 2: Proteins 310 and 301 were covalently immobilized onto MNPs using different pre-concentration buffers. These MNPs were incubated with PSA and biofunctionalized GNPs (respectively 301 and 310). The amount of GNPs/MNPs was calculated from the corresponding SEM images.

3.2. A double-bead sandwich assay

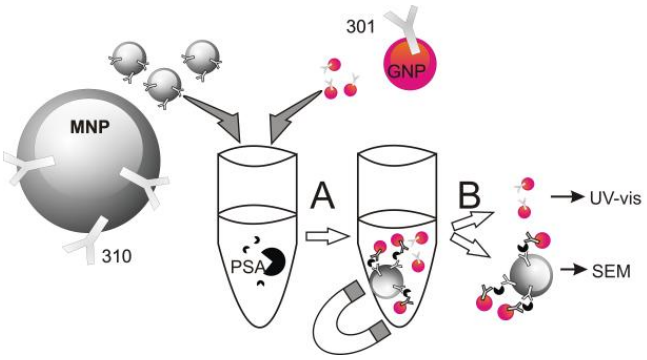

Figure 3: Schematic representation of the performed double-bead sandwich assay. (A: incubation, B: separation)

The biofunctionalized MNPs and GNPs of different sizes were applied in the double-bead assay as schematically shown in figure 2. The unbound GNP-301 fraction was investigated by UV-vis spectroscopy. Upon increase of the PSA concentration, more GNPs-301 will be removed from the supernatant due to sandwich formation.

As a consequence, the plasmon band intensity of the GNPs-301 will decrease relative to the amount of PSA added. As such, this strategy allows quantification of the PSA binding event.

Since the LSPR characteristic of GNPs is dependent on the GNP radius, the effect of the GNP size on the assay sensitivity was evaluated. Hereto, the double-bead assay was performed using GNP-301 of different sizes (20-100 nm). The corresponding dose-response curves are shown in figure 4.

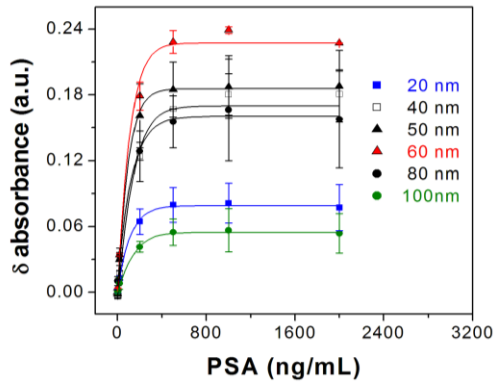

Figure 4: Dose-response curves for the detection of PSA using different sizes of GNPs. The responses are expressed as a decrease in the plasmon intensity.

For all six investigated GNP sizes, a saturation level is obtained at a concentration of 500 ng/mL PSA. It is observed that a high saturation signal gives rise to a steep slope of the dose-response curve in the linear region. Furthermore, it is observed that the smallest (20 nm) and largest (100 nm) GNPs-301 result in the smallest detection signals at saturation, and the lowest sensitivity (< 0.2 pg/mL)$^{-1}$. An optimal detection signal and highest sensitivity was obtained for a GNP size of 60 nm (> 0.7 pg/mL)$^{-1}$. The occurrence of this optimum suggests that there are two opposite trends, one favoring large GNPs and the other favoring small GNPs. These two contributions were evaluated into more detail.

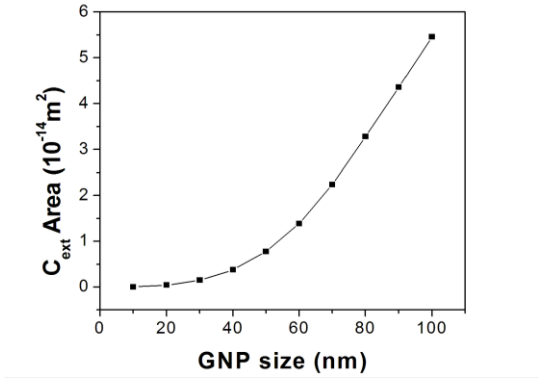

Figure 5: The determined total extinction cross-section for different sizes of GNPs (10-100nm).

First, the total c_{ext} of the GNPs increases almost exponential with the GNPs size as shown in figure 5. This implies that the removal of a single, large GNP in the double-bead assay will cause a larger decrease in the extinction compared to the removal of a smaller one. For this reason, larger particles are preferable for use in the double-bead assay.

Second, smaller beads have a larger surface-to-volume ratio. An increasing amount of bound GNPs-301 is observed with decreasing GNP size. The number of GNPs-301 bound per MNP-310 was also visualized using SEM, as shown in figure 6. Due to their higher electron density, GNPs appear as white dots compared to gray MNPs. The number of GNPs-301 per MNPs-310 at saturation level was roughly estimated from these SEM images (20 nm: 8.1, 40 nm: 2.9, 50 nm: 3.4, 60 nm: 3.2, 80 nm: 1.4, 100 nm, 0.6). These findings indicate that small GNPs can form sandwich structures more efficiently. Furthermore, small GNPs are more efficiently biofunctionalized with proteins compared to larger ones [3]. Another explanation might be the larger mutual steric hindrance upon sandwich formation with large GNPs, as the coverage percentage decreases with increasing GNP size (data not shown). Potentially, this effect could be more pronounced if the immobilization of anti-PSA antibodies is inhomogeneous onto the nanoparticle surface, i.e. assembled in islands.

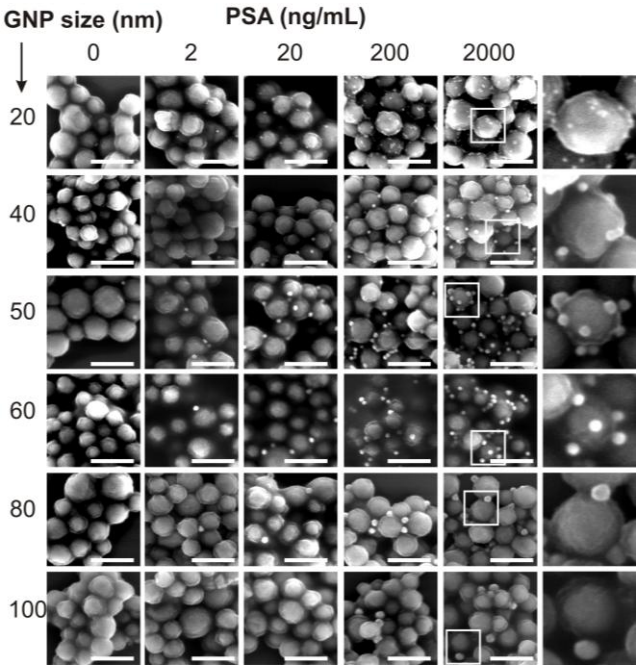

Figure 6: SEM image of the sandwich structures obtained by capturing PSA (0, 2, 20, 200, 2000 ng/mL) using MNP-310 and GNP-301 (20, 40, 50, 60, 80, 100 nm).

3.3. Determination of the assay sensitivity and LOD

As concluded in the previous section, performing the double-bead assay with 60 nm GNPs yields the best detection signals. Therefore these GNPs were used to determine the sensitivity and the LOD of the double-bead assay both in buffer and normal female serum. Normal female serum was chosen to avoid small levels of PSA which can be present in male serum. The experimental results are summarized in figure 7. It can be observed that there is very limited difference between the performances in buffer and serum except at saturation, where the signals in buffer are slightly higher than in serum. Since serum samples contain high amounts of proteins (typically from 50-100 mg/mL), non-specific adsorption of these proteins onto the MNPs may slightly block the specific binding sites for PSA and as such decrease the saturation signal. The corresponding calibration curve for the detection of PSA shows a linear correlation (R^2=0.99) between the amount of PSA and the absorbance of the unbound fraction of GNPs-301 both in HBS and serum. A LOD of 5-8 ng/mL PSA, a sensitivity of 0.7-0.8 $(pg/mL)^{-1}$ and a dynamic range of 3 orders of magnitude were obtained using the proposed double-bead assay. The obtained LOD is lower than the clinically relevant concentration for PSA cancer diagnosis (<10 ng/mL), and is competitive with other detection methods such as surface plasmon resonance (10 ng/mL) or standard ELISA (0.5-4 ng/mL).

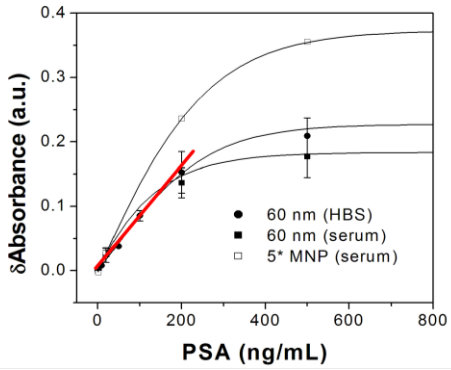

Figure 7: Dose-response curves for the detection of PSA, performed in buffer and normal female serum. The red line corresponds to the calibration curve.

To further decrease the detection limit, one can think of increasing the amount of possible sandwich structures. This can be achieved by increasing the amount of MNPs to efficiently isolate the analyte from the serum sample. Hereto, a preliminary study was performed using a fivefold concentration of MNPs. As can be observed from figure 7, the saturation signal increases by increasing the MNP concentration. This is expected since more gold nanoparticles can be captured in sandwich structures. It can be observed that the sensitivity, and as such the LOD, can be greatly improved. The latter indicates that by simply increasing the concentration of MNPs, the double-bead assay can reach a lower LOD (sub ng/mL (~pM)) and higher sensitivity. This strongly enhances the potential of the double-bead assay for clinical diagnostics.

4 CONCLUSIONS

A simple double-bead assay, using gold and magnetic nanoparticles, was developed for the detection of cancer markers in biological samples. The MNPs were covalently biofunctionalized based on a pre-concentration approach. Not all sizes of GNPs were equally suited to perform the double-bead assay. In fact, an optimal detection signal was found for a GNP size of 60 nm. The occurrence of the optimum was ascribed to two counteracting trends. While the Mie theory favors larger GNPs over smaller ones, it was determined by UV-vis spectroscopy and scanning electron microscopy that larger GNPs were functionalized less efficiently with proteins. Furthermore, by using 60 nm GNPs, clinically relevant concentrations for the detection of PSA, a marker for prostate cancer, were obtained. Moreover, the proposed double-bead assay performed equally well in buffer and serum. In addition, by increasing the concentration of MNPs, a further increase in the assay sensitivity and consequently a lower detection limit can be obtained. Although only the detection of PSA was investigated, the method described in this research is believed to be applicable for the detection of other relevant cancer markers since the biofunctionalization of the nanoparticles is very simple and generally applicable. In addition, it is obvious to consider that the proposed 'double-bead' assay can help to elaborate personal diagnostics due to its simplicity and potential use in miniaturized point-of care systems.

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