Highly Stable and Orientationally Coupled Gold Nanoparticle Antibody Conjugates for Sensitivity Improvement of LFIA

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Gold nanoparticle (AuNP) based Lateral Flow Immunochromatography Assays (LFIAs) have shown wide applicability in medical and research fields [1]. LFIAs are easy-to-handle tools for the investigation of a variety of bio-molecular binding interactions, adequate for point-of-care detection of analytes including pathogens and toxins [2-4]. Innovations for further improving their performance with the help of new bio-conjugation methods remain a challenging task. Here, we have investigated a covalent coupling strategy for preparing gold-antibody conjugates and demonstrated the reactivity of antibodies (IgGs) directionally attached to AuNPs. This strategy helps to resolve limitations arising from random IgG orientation and conjugate instability during fabrication, storage, and usage of traditional LFIAs.

1. INTRODUCTION

The particular interest in AuNPs is motivated by their unique combination of properties. The particles are resistant to oxide formation under ambient conditions, which permits an easy to control attachment of organic molecules onto the gold surface by either exploiting the gold-sulphur bond or through electrostatic interaction. Additionally, gold nanoparticles have special optical properties, brought about by the surface plasmon resonance, which is caused by the collective oscillation of surface electrons induced by incident light [5]. The plasmon band of the AuNPs can be modulated by the nanoparticle size, shape, and the dielectric environment [6].

These properties of AuNPs are brought together in gold-protein conjugates. The protein may be bound to AuNPs covalently, or (more commonly) attached by electrostatic interaction. The specific interaction of gold with sulfur eases the covalent attachment of molecules with thiol functionalities [7].

By coupling a thiol/amino-functionalized polyethylene glycol (HS-PEG-NH_2) linker, (Fig. 1) to Rabbit-IgG (RIgG) we first generated a covalently linked HS-PEG-RIgG polymer adduct. (Scheme 1). Site-specific attachment of the HS-PEG-NH_2 linker to RIgG was achieved by coupling the amino functional group to glycan moieties on the Fc segment of the antibody. The HS-PEG-RIgG adduct was then immobilized onto AuNP via the thiol functional group, while maintaining orientation and activity of the antibody.

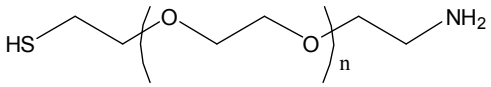
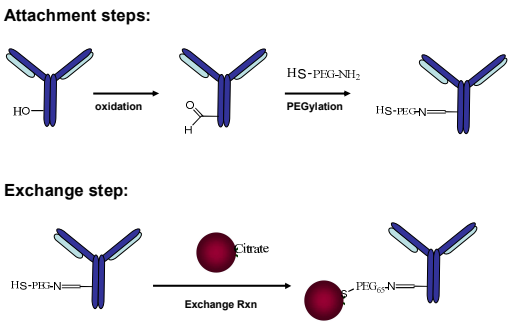


Figure 1: HS-PEG-NH_2 ligand (MW of 3163 g/mol)

Here, we present a simple method to acquire valuable information on the protein coverage and stability of gold nanoparticle conjugates. This method involves the use of Dynamic Light Scattering (DLS) and Lowest Coagulation Concentration (LCC) analysis combined with UV-Vis Spectroscopy. Specifically, we focused on the reactivity, as well as the analysis of surface binding and coverage of IgG-AuNP conjugates coupled via ~3 kD PEG linkers. As reference systems we used citrate- and PEG-stabilized AuNPs, as well as the more commonly used AuNP-IgG conjugates coupled via electrostatic interactions. We related the size increase of AuNPs, measured by DLS after coupling with RIgG, to the approximate number of RIgG molecules per AuNP. This estimation was compared to a Bradford analysis of the AuNPs' protein coverage. Further, the biological activity of the AuNP-PEG-IgG conjugates was investigated using immuno blot assays.



Scheme 1: Illustration of steps in covalent coupling of RIgG to AuNP

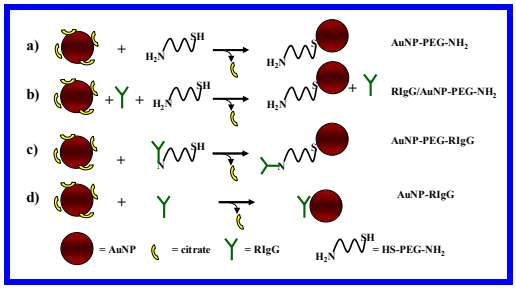
2. MATERIALS AND METHODS

All chemicals used were at least reagent grade and purchased from Sigma-Aldrich, unless otherwise stated. IgGs and Whatman Potran nitrocellulose membranes BA85 were purchased from Sigma-Aldrich, while HS-PEG-NH_2*HCl 3000MW was purchased from Iris Biotech GmbH. Citrate stabilized gold nanoparticles (AuNP) were synthesized following the method described by Frens [8]. The particle concentration of the samples was estimated (using their UV-vis absorption [9]) to be ~16 nM. AuNP core-diameters were ~11 nm, as determined by TEM (Jeol JEM-1011, LaB_6, 100 kV). The AuNP samples were used as prepared for ligand exchange reactions. For the exchange reaction, ligands dissolved in 1xPBS were mixed with the AuNP solutions at high molar excess. The attachment of the HS-PEG-NH_2 linker to IgG was carried out according to a previously published protocol [10] with some modifications, which will be described in detail elsewhere. Purification of the samples was done by repeated centrifugation and resuspension in PBS buffer. A Bradford assay was performed as described in ref. [11] in order to determine the amount of unbound RIgG after the exchange reaction. DLS measurements were carried out using a Malvern Zetasizer Nano ZS ZEN 3600 instrument, UV-vis spectra were recorded using a Cary 50 spectrophotometer.

3. RESULTS AND DISCUSSION

3.1 DLS Measurements

AuNPs are extraordinary light scatterers, a property which is used when characterizing their hydrodynamic diameters by DLS. Using citrate stabilized AuNPs four samples with different surface coatings were prepared via place exchange reactions (Scheme 2): a) HS-PEG-NH_2 was used to obtain sample AuNP-PEG-NH_2. b) A mixture of HS-PEG-NH_2 and RIgG was used to obtain sample RIgG/AuNP-PEG-NH_2. c) PEGylated RIgG (HS-PEG-RIgG) was used to obtain AuNP-PEG-RIgG. d) RIgG was adsorbed electrostatically to obtain AuNP-RIgG. After purification of the samples, they were analysed by DLS measurements.



Scheme 2: Prepared AuNP conjugates

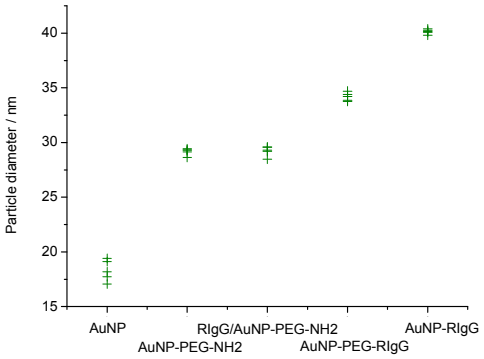


Figure 2: DLS sizes of the samples shown in Scheme 2. The sample AuNP refers to citrate stabilized AuNPs.

Figure 2 shows the DLS-measured sizes of the AuNPs prepared according to Scheme 2. Each sample was measured 5 times. The initial core size of the citrate-protected AuNPs was 11 nm as determined by TEM and their hydrodynamic size was ~18 nm as determined by DLS.

The DLS-sizes of samples AuNP-PEG-NH_2 and RIgG/AuNP-PEG-NH_2 were both ~29 nm. This result indicates that the nature of the moiety covering AuNP-surface was similar in both samples. In the latter case it seems that the surface coverage was strongly dominated by the HS-PEG-NH_2 ligands of the mixture comprising both, PEG ligands and RIgGs. This finding is attributed to the high affinity of the ligand's thiol group to the gold surface, which is stronger than a possible electrostatic interaction between the antibody and the gold surface. In addition, one expects the much smaller PEG ligand reaching the surface of the AuNP faster than the larger RIgG molecules. Compared to the citrate stabilized AuNPs, the increase in size after coupling the PEG-ligand is ~11 nm. This increase in size, which corresponds to a PEG shell thickness of ~ 6 nm, is in reasonable agreement theoretical calculations [12].

In contrast to the AuNPs covered with PEG-linkers, the covalently coupled AuNP-PEG-RIgG and the electrostatically coupled AuNP-RIgG exhibited a larger DLS-measured size, i.e. ~ 34 nm and ~ 40 nm, respectively. AuNP-PEG-RIgG showed an average size increase of about 16 nm compared to citrate stabilized AuNPs, while AuNP-RIgG exhibited a size increase of ~22 nm.

IgGs have a hydrodynamic radius of about 5.5 nm, corresponding to a spherical volume of ~ 700 nm^3 [13]. If we divide the volume of the -PEG-RIgG shell (~1.8 x 10^4 nm^3, sample AuNP-PEG-RIgG) by the hydrodynamic volume of IgG, we obtain as a rough estimate for the number of IgGs per AuNP ~ 26. This value is in remarkable agreement with the result of a Bradford assay, with which we obtained ~27 IgGs per AuNP.

Due to the larger thickness of the IgG-shell in case of the electrostatically coupled IgG (sample AuNP-RIgG, shell volume: ~ 3.0 x 10^4), the estimated number of IgG per AuNP in this sample was somewhat lager, i.e. ~ 43.

3.2 LCC (Lowest Coagulation Concentration)

Citrate stabilized AuNPs are unstable in high salt environment (e.g 1xPBS) and therefore coagulate easily due to deshielding of electrostatic repulsion forces between particles. Due to precipitation coagulation results in loss of the samples' characteristic red color. However, once AuNPs are successfully conjugated with a layer of proteins such as antibodies, the nanoparticles are stabilized in high salt content of buffer solutions. This phenomenon has actually been used as one method to confirm the successful conjugation of proteins to AuNPs [14,15,16]. Furthermore, UV-Vis absorption spectroscopy has been used as a common tool to monitor AuNP agglomeration, because agglomeration causes a red shift and broadening of the surface plasmon resonance of AuNPs.
A logical parameter explored here to determine the coverage of the 11 nm AuNP was the minimum concentration of the stabilizing HS-PEG-RIgG ligand that could confer stability in PBS buffer after ligand exchange. Varied concentrations of HS-PEG-RIgG were used for exchange reactions with a constant volume of known AuNP concentration. This experiment was done by starting with a large ligand excess and then reducing the ligand concentration stepwise until the LCC was obtained. Figure 3 shows a photograph and UV-Vis absorption curves of the samples prepared with HS-PEG-RIgG concentrations within the minimum stability concentration range.

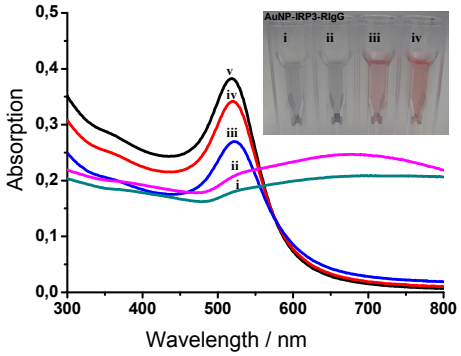
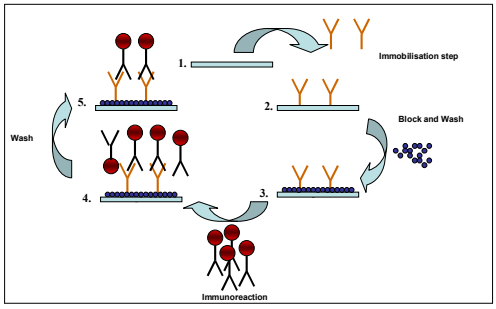


Figure 3: UV-Vis spectra and photograph of AuNP samples mixed with HS-PEG-RIgG ligands at different molar ratios (i-iv: 0.01, 0.11, 1.1, 11 ligand/AuNP, in citrate/PBS (1:1) solution and spectrum v: AuNP in citrate buffer).

The transition from stable (higher ligand concentration) to aggregated particles (lowest ligand concentration) was recognized by the color change in the photograph of figure 3. The ligand/AuNP ratios of the samples shown were 0.01, 0.11, 1,1 and 11 for samples i – iv, respectively. For comparison, the UV-Vis spectrum of the citrate stabilized particles used in these experiments is also presented in figure 3 (v, black curve). As shown by the photograph, samples (iii) and (iv) retained their red color and could visually be described as being stable. However, the corresponding UV-Vis spectrum of the same samples gave a clearer picture, and allowed a fine cut to be made of the solution with the minimum ligand concentration preventing coagulation. Comparing both absorption spectra (iii and iv), it becomes obvious that sample (iii) had lost some particles resulting in reduced optical density and sample (iv) had only changed very little compared to the citrate stabilized AuNP. Thus, for achieving stable solutions of AuNP-PEG-RIgG conjugates ~ 11 HS-PEG-RIgGs per AuNP are required. If we assume quantitative binding of the HS-PEG-RIgG ligand to the AuNP, we conclude that at the lowest coagulation concentration the AuNP surface is covered by ~11 RIgG. This value is somewhat lower than the ratio of ~26 RIgGs per AuNP, determined by the DLS measurement reported above. However, the DLS measurments were carried out with AuNP-PEG-RIgG samples, which were prepared with 1000-fold molar excess of HS-PEG-RIgG ligands. Thus, in case of the DLS measurements it is reasonable to assume saturation of the AuNP surfaces with -PEG-RIgG ligands. In contrast, at the lowest coagulation concentration saturation of the AuNP surface is most likely not achieved.

3.3 Immuno Blot Assays

In order to investigate the immunological activity of AuNP-PEG-IgG conjugates, we prepared AuNP-PEG-GαRIgG (GαRIgG: Goat-anti-Rabbit-IgG) and tested their functionality using immuno blot assays with RIgG as the immobilized antigen. After blocking the NC membranes with BSA, they were immersed into a solution of AuNP-PEG-GαRIgG conjugates and incubated for 2 h. Scheme 3 illustrates the steps involved in the process of the immuno assay.
The results after incubation and drying are shown in figure 4. Spots A, B, C, D and A" on the



Scheme 3: Steps involved in the process of the immuno blot assay.

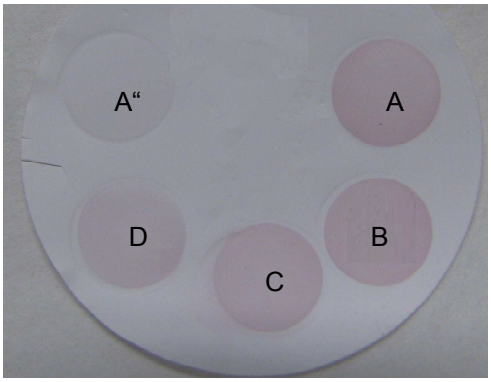


Figure 4: NC membranes with spots of immobilized RIgG after incubation with AuNP-PEG-GaRIgG solution.

NC membrane were prepared with 100 µL solution containing 60 nmol RIgG (spot A), 6 nmol RIgG (spot B), 0.6 nmol RIgG (spot C), 60 pmol RIgG (spot D), and 6 pmol RIgG (spot A”). The complex formation of the immobilised RIgG with the AuNP-PEG-GαRIgG conjugates resulted in red colored spots. As expected, the color intensity diminished with reducing RIgG concentrations. No color appearance was observed on the spot with the lowest concentration, suggesting a lower limit of detection in the range of ~60 pmol.

4. SUMMARY

In this work we have prepared covalently coupled AuNP-PEG-IgG conjugates from citrate stabilized AuNPs (11 nm core diameter) and HS-PEG-IgG ligands. We have investigated the IgG-coverage of the AuNPs using Dynamic Light Scattering (DLS), Bradford analysis, and Lowest Coagulation Concentration (LCC) tests. Our results indicate that the AuNPs are covered by ~ 26 -PEG-IgG ligands, when prepared with a ~1000-fold excess of ligands. At the lowest coagulation concentration the AuNP surface is most likely covered by ~ 11 IgGs. In blot assays the prepared conjugates showed very good immunological activity. Due to their stability and immunological activity we assume that these conjugates are promising candidates for the development of advanced LFIAs.

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