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Properties of surface functionalized iron oxide nanoparticles (ferrofluid) conjugated antibody for lateral flow immunoassay application

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ABSTRACT

In this study, colloidal stability of iron oxide nanoparticles (IONPs) with several acid functionalizations and biocompatible polymer coating were compared for use as labelling agent in lateral flow immunoassay (LFIA). IONPs were synthesized using the precipitation method and peptized using perchloric acid (PA), nitric acid (NA) and citric acid (CA) to form a stable IONPs ferrofluid. Steric stabilization of IONPs using silane polyethelene glycol (SiPEG) was developed to improve biocompatibility and provide spaces for subsequent conjugation process. From the transmission electron microscopy (TEM) images, the sizes of IONPs obtained with different acids peptization were in range of 11–17 nm. The IONPs peptized using citric acid showed the most stable ferrofluid condition at physiological condition with zeta potential value of −49 mV. The LFIA was also developed to examine the conjugation properties of IONPs to mouse anti-human IgG_4 antibody ($M\alpha HIgG_4$). IONPs functionalized with citric acid can be directly conjugated with the $M\alpha HIgG_4$ without the need of SiPEG addition. This is due to the presence of the carboxylic group that acted as a ligand to the extended bond formation with the antibody. Moreover, the conjugation of IONPs with $M\alpha HIgG_4$ was also tested in a LFIA to detect brugian filariasis. The conjugated IONPs-CA without SIPEG showed the optimum detection efficiency within 15 min of assay time.

1. Introduction

The unique physical and chemical properties of iron oxide nanoparticles (IONPs) are of interest for biomedical applications. IONPs have superparamagnetic properties, chemically stable and have low toxicity, thus making them suitable for biomedical applications such as drug carrier, magnetic resonance imaging (MRI) contrast enhancement, and as the labelling agent in immunoassay [1,2]. These biomedical applications require IONPs to be stable in water that is known as ferrofluid. In most biomedical applications, IONPs must be conjugated with antibody, DNA or other biomolecules. Surface functionalization of IONPs is crucial in order to form a stable ferrofluid for the conjugation process. This is due to the high volume to surface area of the IONPs that tend to attract them together and aggregate in order to minimize their high surface energies [3]. Therefore, electrostatic and steric repulsion need to be created between IONPs to prevent agglomeration and produce a stable ferrofluid.

Electrostatic repulsion is strongly influenced by the surface charge of particles and can be created by the acid or base peptization onto the particle surface by lowering the surface tension [4]. Steric repulsion involves formation of coating layer surrounding IONPs normally polymeric based materials to overcome the attractive van der Waals and magnetic potentials, in order to enhance stability of IONPs in water [1]. Several biocompatible polymers have been used for the steric stabilization such as polyvinylpyrrolidone, polyethelene glycols (PEG), poly(vinyl-alcohol) (PVA), and poly(ethylene oxide). Among those, silane polyethelene glycols (SiPEG) is the most promising due to the strong bonding created between silane groups of PEG to IONPs surface and the ability of the silane group to act as the ligand for the subsequent conjugation process [5]. Koehler et al. (2004) [6] used the SiPEG coating to the IONPs, whereby high stability and ligand immobilization on the IONPs surface were obtained.

Since the electrostatic repulsion mechanism is highly pH dependent, much effort had been focused on creating both electrostatic and steric repulsion mechanisms to ensure long term colloidal stability [4,7,8]. Lucas et al. (2007) [4] have employed the peptization of nitric acid on IONPs to avoid the irreversible aggregation of magnetic core before conducting dextran coating to stabilize the steric repulsion. Several studies have reported surface functionalization of IONPs with citric acid, then directly attached it with protein, drug or dye by exploiting the carboxyl group presence on the surface of the nanoparticles [3,9]. Recently, Deb et al. (2011) [10] fabricated citric acid functionalized IONPs using

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co-precipitation method. However, most of the previous studies applied heat during the reaction. Furthermore, Cheng et al. (2009) [11] have synthesized carboxyl dextran-coated IONPs through the flocculation of cluster using poly(vinyl-alcohol) (PVA) and use the electrostatic repelling effect of citric acid to remain stable in aqueous solution. The presence of carboxyl group facilitates the IONPs for the subsequent conjugation process with a monoclonal antibody and has potential to be used as a molecular probe in cancer diagnosis.

Lateral flow immunoassay (LFIA) is also known as the immunochromatographic assay or strip test that offer a rapid, highly sensitive, fast result and low cost [12]. Currently, LFIA has gained wide acceptance in the determination of food contamination [13], detection of environmental toxic pollutant [14] and medical diagnosis [15]. There are numerous coloured particles used in the LFIA as the labelling agent such as gold nanoparticles [13,14], iron oxide nanoparticles [2], fluorescence dyes [16] and carbon nanoparticles [17]. In LFIA, gold nanoparticles have been widely employed as a marker due to the intense colour and the stability of gold in liquid or dry form [18]. However, the rising price of gold has lead to the search for new materials. To address this need, in the present study IONPs is used as a labelling agent in the LFIA.

Notably, many studies have reported on the surface functionalization of IONPs to form stable ferrofluid and provide ligand for the subsequent conjugation [3,19]. There are few studies that compare the properties of IONPs stabilized with different types of acids thus in the present study this aspect was investigated [4,20]. Moreover, this study also embarks on combining electrostatic repulsion and steric repulsion mechanisms to obtain a stable ferrofluid. Nitric acid, perchloric acid and citric acid were peptized on IONPs to provide the surface charge and stability to the IONPs in water. Then SiPEG was coated to IONPs to increase the hydrophilicity and acts as the ligand for conjugation to the antibody. Most previous studies on conjugation of IONPs used crosslinkers to perform the covalent bonding of IONPs to the antibody [19]. However in this study, the stable IONPs (ferrofluid) were directly conjugated to the MαHIgG_4 and the performance of the conjugated IONPs-MαHIgG_4 as the labelling agent in LFIA was assessed. The conjugation properties of IONPs-MαHIgG_4 were compared to obtain the best conjugation approach that works well in labelling LFIA. The immobilization of MαHIgG_4 to the silane group of IONPs coated with SiPEG was compared with the immobilization of MαHIgG_4 to the IONPs peptized with acids. MαHIgG_4 was also immobilised directly on the surface of functionalized IONPs to obtain the conjugated IONPs-MαHIgG_4 as the labelling agent in LFIA. In addition, sensitivity and specificity analysis of the conjugated IONPs- MαHIgG_4 as the labelling agent in LFIA were evaluated systematically.

2. Materials and methods

2.1. Synthesis of iron oxide nanoparticles

IONPs were precipitated by adding 0.3 M Iron (II) chloride ($FeCl_2$, Sigma–Aldrich) and 1 M sodium hydroxide (NaOH, Fisher Scientific) simultaneously into 600 ml of water and the reaction was maintained at pH8 using a titrator (842 Titrando, Metrohm). The synthesis was carried out in a bioreactor under nitrogen atmosphere. Then, oxidation of the precipitates formed was carried out using 1.7 M hydrogen peroxide (H_2O_2, Fisher Scientific). The solutions changed colour from milky green to black after the oxidation which indicated the formation of IONPs. After oxidation, the reaction was allowed to completely crystallize for 2 h under continuous stirring. Subsequently, the precipitates were collected and the supernatant was discarded using magnetic separation, and then washed with water. The solution was divided into three beakers and 3.5 M of perchloric acid (PA, Merck), nitric acid (NA, Merck) and citric acid (CA, Merck) were added into each beaker, respectively and left overnight. Second peptization was carried out the next day using the same concentration of acids and washed two times using 1.7 M concentration of each types of acids. The IONPs (IONPs-PA, IONPs-NA and IONPs-CA) produced were collected and dispersed in water. The morphology, particle size and distribution were investigated using a transmission electron microscope (TEM) (Philips CM12.Version 3.2). Particle size of IONPs was measured using ImageJ software from TEM images, phase identification of IONPs was determined using X-ray diffraction (XRD) (P8Advan-Bruker with Cu-K_α radiation source) and the concentration of Fe was determined using Beer's law from UV–Visible near-infrared spectrophotometer (UV–Vis) (UV-3600, Shimadzu). Zeta potential was measured using Zetasizer (Malvern, Nanozs).

2.2. PEGlation of iron oxide nanoparticles

IONPs-PA, IONPs-NA and IONPs-CA were coated with various amounts of SiPEG (0.52 μl, 1.0 μl, 2.0 μl and 3.0 μl) by pipetting the SiPEG (ABCR) solution into the ferrofluid and rotated overnight. The electrophoretic mobility of the IONPs was measured using the Zetasizer Nano ZS (Malvern). The ferrofluids were filtered using 0.22 μm syringe filter (Milipore) to separate the IONPs aggregates. The filtration efficacy of IONPs was measured to obtain the optimum amount of SiPEG coating that produce stable and less aggregates of IONPs. The higher the filtration efficacy value, the lesser are the aggregated particles in the ferrofluid. The filtration efficacy can be calculated based on the following equation [21]:

$$\text{Filtration efficacy}(\%) = \frac{\text{Fe(III)concentration after filtration}}{\text{Fe(III)concentration before filtration}} \times 100$$

2.3. Conjugation of iron oxide nanoparticles to monoclonal antibody

The concentration of ferrofluid was diluted to 1 M and pH of the IONPs coated with SiPEG and IONPs peptized with acids was adjusted to pH 7. This was due to the isoelectric point (IEP) of MαHIgG_4 was at pH 7. Then, 20 μl of 1 mg/ml MαHIgG_4 was added and the mixture was incubated for 1 h. Subsequently, 1% of bovine serum albumin (BSA-Sigma–Aldrich) was added as a stabilizer and to block the unconjugated areas from unspecific binding. The IONPs conjugated MαHIgG_4 was centrifuged at 10,000 rpm for 10 min to remove the unconjugated antibody and washed using 1% BSA. Finally, the IONPs conjugated antibody was collected and suspended in 50 μl of 1% BSA.

2.4. Development of lateral flow immunoassay (LFIA)

LFIA was developed using a nitrocellulose membrane card (NC card, Milipore) as a chromatography matrix and the absorbent pad as a liquid sink. Isoflow dispenser (Imagene Technology) was used to line the NC card. The human IgG_4 (0.7 mg/ml) was lined as a control line and 1 mg/ml of *Brugia malayi* recombinant antigen (*Bm*R1) was lined as the test line. Then, the NC card was dried in an oven at 37 °C for 2 h. Thereafter, the NC card was treated with a blocking solution comprising Western blot blocking solution (Roche, Germany), tris–hydrocloric (Tris–HCl, Sigma–Aldrich) and sodium chloride (NaCl, Sigma–Aldrich). The NC cards were then dried overnight before cutting to 5 mm strips for testing.

2.5. Sensitivity and specificity testing of conjugated IONPs- MαH IgG4 with LFIA

The sensitivity of conjugated IONPs-MαHIgG_4 was tested with LFIA line with human IgG_4 as the control line. The conjugated IONPs-MαHIgG_4 was diluted by mixing 30 μl of 0.01 M PBS (pH 7.2) with 5 μl of IONPs-MαHIgG_4 into a well of a microtitre plate. Then, 5 mm strips were cut from the NC card, the absorbent pad was attached and then dipped into the well which contained the diluted conjugated IONPs-MαHIgG_4. Then, the background colour of the unbound IONPs was washed by dipping in a well containing 0.01 M PBS. The presence of one brown line proved that the binding occurred between IONPs and the MαHIgG_4. The specificity testing of conjugated IONPs-MαHIgG_4 was conducted using positive and negative serum samples, i.e. brugian filariasis patient's serum and normal healthy person's serum samples, respectively. Human IgG_4 was used as the control line and the *Bm*R1 recombinant antigen as the test line. First the strip was placed in a well containing the diluted serum sample, followed by dipping in a well containing the diluted conjugated IONPs-MαHIgG_4, and followed by placing in a well containing 0.01 M PBS.

3. Results and discussions

3.1. Synthesis of iron oxide

Peptization of IONPs using various acids is very important in order to form a stable ferrofluid. Fig. 1(a) shows the IONPs collected after synthesis which was not stable in water and settled to the bottom due to agglomeration while Fig. 1(b–d) shows IONPs collected after peptization was stable. This phenomenon occurred because of the Van der Waals, magnetic and electrostatic forces presence between IONPs that led to agglomeration [22]. After peptization with acids, the presence of electrostatic force between IONPs prevents agglomeration of particles. Fig. 2 shows the XRD