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Original Research Article

Direct Immobilization of Coagulation Factor VIII on Au/Fe_3O_4 Shell/Core Magnetic Nanoparticles for Analytical Application

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HIGHLIGHTS

- Magnetic nanoparticles are the most attractive nanostructures in biomedical and bio-analytical fields.
- The protein coating on MNPs has been found to have wide clinical and analytical applications.
- Coagulation factor VIII (FVIII) is a valuable therapeutic human protein in the market.
- Attachment of a large protein like F VIII to GMNPs is affected by various environmental factors.

ABSTRACT

Protein-coated nanoparticles have diverse applications in biomedical science. The protein hydrophobic domains or surface electrostatic charge conducts adsorption of proteins to different surfaces. This property can be customized to immobilize specific molecules on solid supports for experimental screenings or purification processes. To develop highly selective affinity ligands—such as aptamers—against specific protein targets, protein-coated magnetic particles have been successfully applied. This approach could be highly efficient in affinity ligand development against coagulation factor VIII.In this study, magnetic nanoparticles were prepared by co-precipitation method and, then, a gold coating was run on the MNPs' surface. The gold coating could add some attractive specifications to the protein immobilized nanoparticles during the aptamer selection process, such as simultaneous affinity determination of aptameric oligonucleotides by fluorescence-based methods. The gold surface has been indicated as a specific feature for covalent binding to the sulphur functional groups of various molecules. In proteins, sulphur units of cysteine or methionine might be bound covalently to the gold surface. In addition, nonspecific and non-covalent attachment of proteins to the gold particles may be performed. Therefore, a series of samples containing different mass ratios of protein to gold magnetic nanoparticles (GMNPs) were evaluated to find the best conditions for coagulation factor VIII immobilization. The results showed that the best condition for high coating efficiency was 48 h incubation at 4 ºC of protein and GMNPs with a mass ratio of 0.5% in PBS 25mM, with pH=7 as binding buffer.

Introduction

Nano-materials are promising structures in various fields of the biological sciences. Nanostructures with different identities for different applications have been developed, using a wide variety of starting materials (metals and biological and chemical polymers). Among these, magnetic nanoparticles (MNPs), especially Fe_3O_4 nanoparticles, are the most attractive nanostructures in

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the biomedical and bio-analytical fields because of their specific characteristics, such as superparamagneticity, biocompatibility, non-toxicity, and ease of synthesis (Wu et al., 2015; Zeinali, 2015).

Nowadays, magnetic nanoparticles are being extensively studied for their biomedical applications, such as magnetic separation, cell labelling, cellular therapy, drug delivery, hyperthermia treatment, and magnetic resonance imaging (MRI) (Colombo et al., 2012). MNPs are among the most valuable particles in the protein science researches, as they are easily handled with an external magnet due to their paramagnetic feature. In addition, it is possible to change their surface characteristics by adding chemicals, metals such as gold, silica, acrylamide and agarose, or biological moieties such as streptavidin, monoclonal antibodies, aptamers, peptide ligands and lipids. The modifications result in specific applications.

A modified type of magnetic nanoparticles is core-shell magnetic nano-composites that attract significant attention due to their extra features in addition to magnetic nano-structures and, now, they are being extensively investigated for a wide range of applications (Chatterjee et al., 2014). Gold-coated MNPs (GMNPs) are one of the promising nanosystems in this regard. The GMNPs were developed by the reduction of a gold salt such as gold chloride on the surface of pre-formed magnetite nanoparticles (Wang et al., 2008). GMNPs have been particularly designed for biomedical applications ranging from magnetic bio-separation to bio-imaging and targeted therapy (Bao et al., 2009; Qiu et al., 2010; Tamer et al., 2010).

Protein coating on MNPs or magnetic nano-composites have been widely investigated for numerous clinical and analytical applications (Xu et al., 2014). The physical and chemical features of nano-materials, particularly their surface characteristics, affect their interaction with proteins. Different proteins have been studied in combination with MNPs to achieve medicinal or bio-analytical applications. For example, a study evaluated the adsorption of bovine serum albumin, myoglobin, and cytochrome C to lipid-coated gold nanoparticles, and found that all three proteins form adsorption layers consisting of an irreversibly adsorbed fraction and a reversibly adsorbed fraction (Kaufman et al., 2007).

Coagulation factor VIII (FVIII) is a valuable therapeutic human protein with a molecular weight of about 300 KDa that has been used in the therapeutic regimen of haemophilia patients (Gouw et al., 2013). This therapeutic protein has been supplied from two sources: plasma or recombinant production. Regardless of the source that was used as starting material, protein purification process has a significant impact on the quality of the final biopharmaceutical product. Nowadays, chromatography-based methods, particularly affinity chromatography, have a special position in laboratory-scale and industrial protein purification (Ayyar et al., 2012; Hage et al., 2012). In the purification process of coagulation proteins, affinity chromatography is a highly efficient method (Burnouf, 2007; Gouw et al., 2013; Rodrigues et al., 2015; Wang et al., 2003).

In this study, immobilization of coagulation factor VIII on gold-coated magnetic nanoparticles (GMNPs) was evaluated in order to prepare a protein-fixed solid support for the following analytical and investigational approaches. Firstly, a core/shell nanostructure of gold on iron magnetic nanoparticles was prepared and, then, the best condition for the immobilization of plasma-derived coagulation factor VIII on the nanoparticles was assessed.

Materials and Methods

Chemicals

The iron salts—$FeCl_3 \cdot 6H_2O$ and $FeCl_2 \cdot 4H_2O$, and PVA 15000—were supplied by Merck chemicals (Germany). Electrophoresis chemicals, tetraethyl orthosilicate (TEOS) and Gold (III) chloride were purchased from Sigma (USA). Haemoctin® 500 (Biotest, UK) was used as plasma coagulation factor VIII sample. Better Bradford Protein Assay Kit of BioBasic (Canada) was used for protein quantification.

Preparation of Fe_3O_4 @ SiO_2 @ Au core/shell magnetic nanoparticles

Preparation of Fe_3O_4 nanoparticles

The mixture of $FeCl_3 \cdot 6H_2O$ (1.3 g, 4.8 mmol) in water (15 mL) was added to the solution of polyvinyl alcohol (PVA 15000) (1 g), as a surfactant, and $FeCl_2 \cdot 4H_2O$ (0.9 g, 4.5 mmol) in water (15 mL), which was prepared by completely dissolving PVA in water followed by the addition of $FeCl_2 \cdot 4H_2O$. The resultant solution was left to be stirred for 0.5 h in 80 °C. Then NaOH (1.0 mol/L) was added drop-wise, with vigorous stirring, to produce a black solid product when reaction media reached pH 10. The resultant mixture was heated on a water bath for 2 h at 60 °C and the black magnetite solid product was separated, washed with ethanol three times, and then dried at 80 °C for 10 h.

Preparation of Fe_3O_4@SiO_2 core–shell

The core–shell Fe_3O_4@SiO_2 nanospheres were prepared by a modified Stober method (Esmaeilpour et al., 2014). Briefly, Fe_3O_4 (0.50 g, 2.1 mmol) was dispersed in the mixture of ethanol (50 mL), deionized water (5 mL) and tetraethoxysilane (TEOS) (0.20 mL), followed by the addition of 5.0 mL of NaOH (10% w/v). This solution was stirred mechanically for 30 min at room temperature. The final product—Fe_3O_4@SiO_2—was separated by an

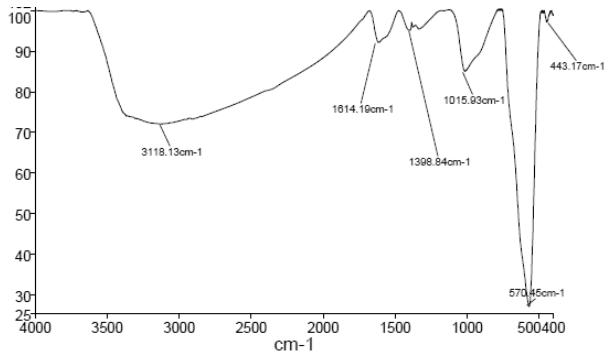

Figure1. FTIR spectra of SiO2 coated Fe3O4 magnetic nanoparticles.

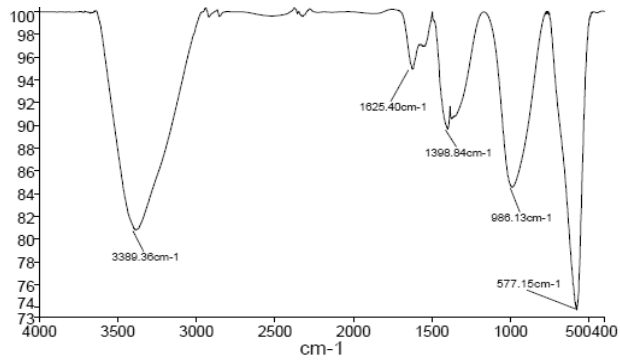

Figure 2. FTIR spectra of gold coated Fe3O4@SiO2.

external magnet, and washed with deionized water and ethanol three times, and then, dried at 80 ºC for 10 h.

Preparation of Fe3O4@SiO2@Au

A 200 mg sample of the Fe_3O_4@SiO_2 was added in 20 mL water with 100 µL of $AuCl_3$ (200 mg/mL), then 3 ml of $NaBH_4$ (1.0 mol/L) solution was added drop-wise with vigorous stirring for 2h. Finally, the product—Fe_3O_4@SiO2@Au nanoparticles—was separated by an external magnet and washed three times with deionized water and ethanol. At the end, gold magnetic nanoparticles were dried at 80 ºC for 10 h.

Protein coating on gold magnetic nanoparticles

Haemoctin as the source of coagulation factor VIII contains 5 mg protein as a lyophilized powder in each vial. Each vial was reconstituted by adding 5 mL of calcium supplemented Tris buffer (pH 7.4, Tris-HCl 40 mM, $CaCl_2$ 5 mM). Different mass ratios of protein to GMNPs were prepared and incubated in different conditions. After incubation time, GMNPs were separated from incubation mixture using an external magnet. Two steps of particle washing with buffer were run. The total supernatant from three runs of particle separation was collected and, then, the supernatant residual protein was measured by Bradford assay. Bradford assay was applied to measure the total protein, based on a serum albumin standard curve (Kruger, 2009).

Electrophoretic study of the protein-coated gold magnetic nanoparticles

Protein coating on GMNPs was evaluated by Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, sample buffer 1X (200 µL; 50 mM Tris-HCl pH 6.8, 2.5 % SDS, 0.002 % Bromophenol Blue, 5 % β-mercaptoethanol, 10 % glycerol) was added to each sample of protein-coated GMNPs and the contents were boiled for 10 min on a water bath. Then, particles were separated by external magnet and supernatant sample (40 µL) was applied to the SDS-PAGE (4 % stacking gel–10 % resolving gel).

Instrumentation

Magnetometery

Magnetic properties were evaluated by vibrating sample magnetometer-BHV-55 model (Riken Denshi Co., China).

Fourier transform infrared (FT-IR) spectroscopy

Fourier transform infrared (FT-IR) spectra were recorded (Perkin Elmer spectrometer, USA) at room temperature.

UV–visible spectroscopy

UV–visible (UV–vis) absorption spectroscopic measurements were recorded using ScanDrop (Analyticjena, Germany).

Particle size analysis

Particle size analysis was performed by Dynamic Light Scattering (DLS) method using ZetaSizer Nano ZS-ZEN3600 model (Malvern, UK). Samples were analysed in aqueous solution at room temperature.

Results

Fe_3O_4@Au core-shell magnetic nanoparticles

Silica coating was considered to improve the stability of the Fe_3O_4 magnetic nanoparticles prepared. Then, gold shell was added to the silica-coated Fe_3O_4 MNPs. The particle size of final core/shell nanocomposites was 169 nm (PdI=0.231) compared to 203 nm of SiO_2@Fe_3O_4 MNPs. Gold coating resulted in an elevation of zeta

Table 1. Protein coating efficacy under three conditional factors.

Protein/GMNP Mass ratio	% protein binding				Incubation time
	PBS; pH=7	Tris-HCl; pH=6	Tris-HCl; pH=7	Tris-HCl; pH=8	
0.5	15	14	12	10	2
0.5	18	16.5	13	11.5	4
0.5	26	21.6	20.5	19	8
0.5	52.4	49	36	38	16
1	12	10	9.5	8	2
1	14	14	11	10	4
1	20.6	19.3	16.5	17	8
1	38	39	26.2	19	16
2	13	12.3	11.6	10	2
2	16	14.9	12	11.3	4
2	25.3	25.1	23	20.6	8
2	46	36	29.2	21.3	16
0.5	86	69	58	32.2	48
0.5	75	65	49.3	29.8	72

Note: Temperature was set at room temperature for 2 & 4 hours incubation and 4ºC for 8 h and more incubation time

potential to -1.46 mv.

Coatings of silica and gold were confirmed by FTIR (Figure 1). The absorption peak of around 580 cm^{-1} indicated the stretching vibration mode of Fe-O and Fe-O-Si bonds, while the absorption peak of 1000–1150 cm^{-1} typically referred to the stretching vibration of Si-O-Si and Si-OH groups. A shift to the lower wavelength for the absorption peak (1015 to 986 cm^{-1}, Figure 2) confirmed the gold coating on SiO_2 shell of MNPs. Moreover, the peaks 1614 cm^{-1} and above 3300 cm^{-1} were caused by the adsorbed water in the sample.

The magnetic properties of nanoparticles were measured by VSM at room temperature (Figure 2). Both MNPs and gold-coated MNPs showed a typical super paramagnetic manner. The phenomenon of hysteresis was not found, and the magnetization and demagnetization curves were coincident.

Protein coating on GMNPs

Coating of coagulation factor VIII on GMNPs were evaluated on the basis of three factors: included protein to GMNPs mass ratio, time, and buffering agent. The binding efficiency was calculated in accordance with the percentage of residual protein in supernatant, after magnetic separation, compared to the starting protein mass (Table 1). Among different protein to GMNPs mass ratios that were evaluated in this study, the best condition was achieved in 0.5% (w/w). In samples with high protein content, the nanoparticles were extensively aggregated. This might be the result of the large size of coagulation factor VIII, which would simultaneously attach to more than one nanoparticle. According to the different binding buffers with different pH that were studied (Tris-HCl 40mM, pH=6-8; PBS 25mM, pH=7), the PBS buffer with pH=7 showed better binding between FVIII and GMNPs (≥52% during 16 h incubation). In addition, results showed that the incubation time should be extended to 16 hours and more, in order to attain an acceptable binding efficiency. Accordingly, the incubation temperature was set at 4 ºC, due to the protein instability in long-time incubation at room temperature. Moreover, the least volume of reaction (100 µL) exhibited higher binding efficiency than 200–500 µL incubation volumes. Finally, the binding of FVIII on the GMNPs was evaluated by SDS-PAGE (Figure 3) that confirmed the presence of FVIII bound to the prepared MNPs. The best condition for high coating efficiency (86% binding) was achieved after 48 h incubation at 4 ºC of protein and GMNPs with mass ratio of 0.5% in PBS 25mM with pH=7 as binding buffer. The decrease in the binding percentage after 72 h incubation could be the result of protein desorption from the particles, which indicated some instabilities in protein attachment to GMNPs.

FTIR analysis of protein-coated particles revealed that the protein F VIII was coated on the GMNPs (Figure 5). Absorption peak 2984 cm^{-1} indicates the alkyl group-CH_2, 1629 cm^{-1} for C=O of amide I, 1552 cm^{-1} for amide II and 3189 cm^{-1} for hydroxyl groups confirmed the protein coating on GMNPs.

Particle size was increased after protein coating on GMNPs and the average size of 619 nm (PdI=0.244) was recorded. The zeta potential of protein-coated nano-composites was recorded as -8.32 mv.

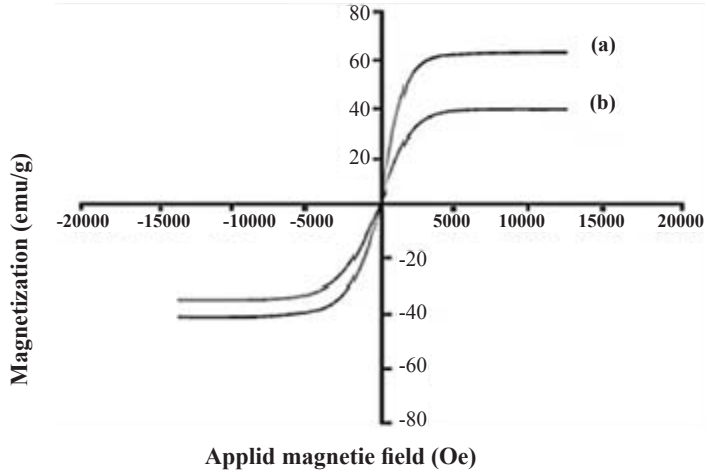

Figure 3. Magnetization curves at 300 K for (a) Fe3O4, (b) Fe3O4@SiO2@Au.

Discussion

Magnetic nanostructures have been developed by various procedures, which involve physical, chemical and biological methods. These methods involve precipitation from solution, co-precipitation, high temperature decomposition of organic precursors, gas-phase deposition, electron beam lithography, sol-gel synthesis, oxidation method, hydrothermal reactions, aerosol/vapour phase method and microbial incubation. Co-precipitation method may be the most promising one due to its simplicity, productivity, ease of implementation and need for less hazardous materials (Indira and Lakshmi, 2010; Xu et al., 2014). Naked iron-based magnetic nanostructures are chemically and physically unstable. Therefore, coating of iron MNPs with natural or synthetic polymers has been used and evaluated in this respect, and exhibited promising results (Turcheniuk et al., 2013; Xu et al., 2014). In this study, silica coating was applied to stabilize iron-based magnetic core. The coating resulted in a more negative zeta potential and was confirmed by FTIR. The results of magnetometery indicated that coating of Fe_3O_4 significantly decreased the magnetization property. However, the separation of the GMNPs by applying an external permanent magnet could make the recovery of the nanoparticles straightforward.

Coagulation factor VIII is one of the valuable therapeutic proteins that has been purified from plasma or recombinant source by different chromatography processes, especially affinity chromatography (Burnouf and Radosevich, 2001; Rodrigues et al., 2015). Aptamers—as a selective affinity ligand—could be applied in protein purification

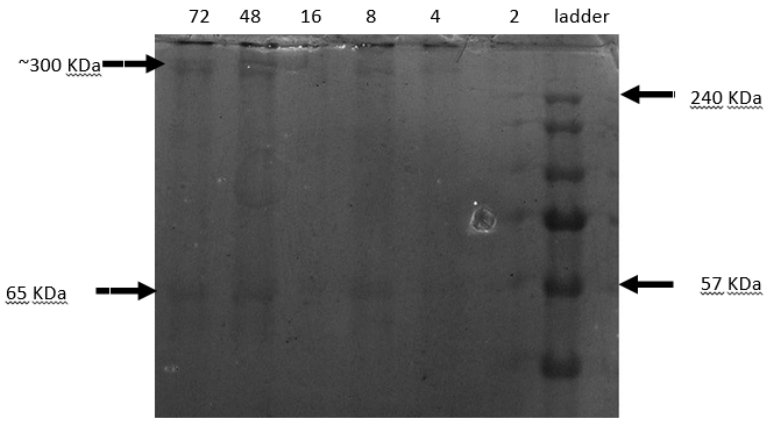

Figure 4. Electropherogram of FVIII coated GMNPs on 10% SDS-PAGE; from right to left: wide range protein ladder (10-250KDa), 100 µL reaction 2, 4, 8, 16, 48 and 72 hours incubation. The band above 250KDa is the FVIII (~300KDa) and the other band around 65 KDa is the contaminating coagulation factor II in Haemoctin.

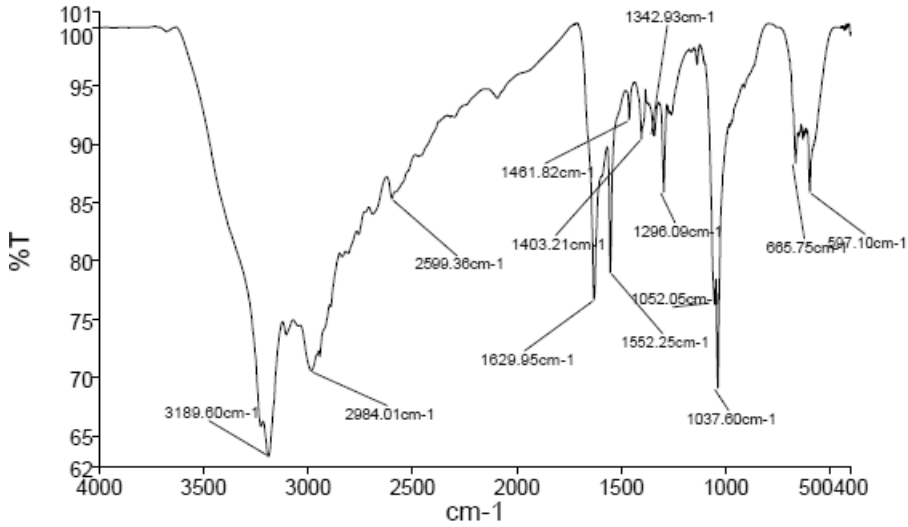

Figure 5. FTIR spectum of FVIII coated GMNPs

(Lim,et al.,2013; Lönne,et al.,2015; Qiao,et al.,2015; Wang,et al.,2015). The magnetic nanoparticles provide good solid support for protein specific aptamer selection (Yang et al.,2016). Accordingly, we tried to immobilize coagulation F VIII on GMNPs in a way that could be applied for specific aptamer selection. F VIII Protein attachment to the gold surface of GMNPs was performed by at least 16 hours' incubation of F VIII with GMNPs in 4 ºC, regarding less stability of the protein at room temperature (Wang et al.,2003). The results confirmed that the amount of coagulation factor VIII coating on the GMNPs were significantly low in the short incubation time. The large size of coagulation factor VIII and the probability of only one sulphur group being present on the protein surface (according to the computational modelling by visual dynamic modelling 'VMD' software) might result in slow protein binding to GMNPs. Protein coating resulted in some particle size growth, which might be the result of protein-assisted aggregation of nanoparticles. In addition, according to the F VIII instabilities and also lack of stable attachment of the protein to GMNPs (as seen by a reduction in the binding percentage after 72 h, compared to 48 h incubation), it should be considered that the protein-coated GMNPs have to be used immediately once the preparation was completed.

Conclusion

In the case of magnetic nanoparticles, simple handling, various surface modifications and large surface area make them one of the most promising analytical materials. Gold nanoparticles have also been extensively studied in biomedical and bio-analytical fields of application. Coating of coagulation factor VIII on magnetic nanoparticles can provide a valuable protein-coated solid support for further analytical applications. The results of this study showed that the direct attachment of FVIII onto the surface of GMNPs could be achieved, but this encouraged aggregation of particles. Therefore, the final product ended with protein-coated GMNPs in the micrometre range of size. In addition, the protein was non-specifically adsorbed on the GMNPs' surface. Consequently, it would be better to add a linker within in order to conduct the protein binding in a defined orientation.

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Competing Interests

All the authors confirmed that there is no conflict of interest in this study.

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