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Surface Functionalization of Magnetic Nanoparticles for the Enrichment and Detection of Albumin in Urine

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Abstract: In recent years, magnetic nanoparticles (MNPS) and their use in biomedical and biotechnological applications have received a lot of attention. In this study, the surface of the iron oxide magnetic nanoparticles was functionalized to achieve high affinity and sensitivity in targeting a specific molecule. The immobilization of avidin on the functionalized MNPs was done in preparation for the coupling of biotinylated aptamers to target human serum albumin for the detection of microalbuminuria. The structural information of functionalized magnetic nanoparticles was characterized using transition electron microscopy (TEM), Fourier transform infrared spectroscopy (FTIR), and thermogravimetric analysis (TGA). The ultra-high performance liquid chromatography (UPLC) was used to detect the trace albumin in spiked buffer samples. It shows that for 0.01 ppm HSA concentration, an uptake of 55.03 ±1.15 % and an LOD of 1 pbb are observed.

Keywords: Iron (II, III) oxide nanomagnetic particles, Strepavidin, Immobilization, Nanoparticle functionalization, Human serum albumin.

1. Introduction

Human Serum Albumin (HSA) is multifunctional and is the most abundant protein in human blood plasma. The multifunction of HSA is associated with its structure that allows to bind and transport a number of metabolites such as fatty acids, metal ions, bilirubin and some drugs [2]. It is normally present at high concentrations in the human blood (i.e., >30 mg/mL). However, albumin should not exist in urine beyond a clinically normal threshold value of 30 μg/mL. As in the case of kidney damage, small amounts of albumin that leak into the urine would lead to a condition known as the microalbuminuria [3].

Microalbuminuria (MA) is defined as a condition characterized by having levels of albumin ranging from 30 to 300 mg in a 24-h urine collection [4]. It is an established risk marker for the presence of cardiovascular disease and predicts progression of nephropathy when it increases to frank microalbuminuria >300 mg/d (>200 μ g/min) [5].

Early stages of kidney disease can be diagnosed using various tests performed on blood pressure, serum creatinine, as well as urine albumin. [3]. The

microalbuminuria testing or a simple test that can measure the amount of albumin in the urine can be done by using a diagnostic tool such as a urinary dipstick. However, standard urinary dipsticks can only detect albumin at levels greater than 300 mg/24 hours. To address this issue, the sensor technology has provided an alternative method to detect albumin in a small quantity sample by using magnetic nanoparticles (MNPs).

Magnetic nanoparticles (MNPs) is one of the tools recently developed to purify or separate specific proteins from biological matrices. The iron oxides magnetite (Fe_3O_4). MNPs are preferred because of their greater saturation magnetization [7]. These MNPs without coating, tend to aggregate to form large clusters. Therefore, it is necessary to functionalize their surface to apply its usage in the fields of biotechnology and to prevent agglomeration. Aptamers have various advantages as recognition elements but no study has been done in the use of functionalized magnetic nanoparticles (MNPs) for the detection of microalbuminuria. Hence, surface functionalized MNPs have been developed for the detection of microalbuminuria using standard solutions of albumin to mimic a real urine sample. This paper primarily aims to functionalize the surface of the iron (II, III) oxide magnetic nanoparticles by adding functional groups and immobilizing it with aptamer for the sensitive and selective detection of albumin.

2. Materials and Methods

2.1. Reagents

The 5 % glutaraldehyde solution used was manufactured by Sigma-Aldrich. The human serum albumin (HSA), iron oxides magnetite (Fe_3O_4), tetraethyl orthosilicate (TEOS), aminopropyltriethoxysilane (APTES), were purchased from Sigma-Aldrich. The aptamer (H-APT1) used was manufactured by Sangon Biotech (Shanghai) Co., Ltd.

2.2. Functionalization of Iron Oxide Nanoparticles

2.2.1. Preparation of Silica-coated Magnetic Nanoparticles

Iron oxide (II, III) particles were weighed with a weight of 0.5 grams on a conical tube. The particles were washed with fifteen milliliters (15 mL) of ultrapure water and adding 200 μL 25 % sodium hydroxide (NaOH) solution was added drop by drop until the solution became pH 11. The mixture was sonicated for about 30 minutes to disperse the MNPs in the alkaline solution. The supernatant and the MNPs were separated through magnetic decantation. tetraethyl orthosilicate (TEOS) with different volumes (1, 2, 4, 6, 8 mL) was added to the particles. To the mixture prepared, one mL of 25 % sodium hydroxide NaOH and 40 mL ethanol was added. The mixture in each tube was mechanically stirred for 10 hours at 350 revolutions per minutes (rpm). After mixing, the supernatant in each tube was removed using an external magnetic field. The silica-coated MNPs were washed with ultrapure water and ethanol (three times) and dried it in a vacuum oven at 70 ºC overnight.

2.2.2. Surface Functionalization of Silica-coated Magnetic Nanoparticles

Onto the dried silica-coated MNPs, two mL of aminopropyltriethoxysilane (APTES) was added to introduce amine groups on the surface of the treated MNPs. One milliliter of 25 % sodium NaOH and 40 mL ethanol was added to the mixture. The mixture in each tube was mechanically stirred for 10 hours at 350 rpm. The supernatant was removed through magnetic decantation. The separated amine functionalized MNPs were rinsed with ultrapure water and ethanol (three times) and dried in a vacuum oven at 70 ºC.

2.3. Immobilization of Functionalized Magnetic Nanoparticles

2.3.1. Activation of Amine-functionalized Magnetic Nanoparticles

Ten milliliters (10 mL) of phosphate buffered saline (PBS) solution were added to the amine-functionalized MNPs. The Coupling buffer was added to the mixture until the final volume reached 50 mL and the mixture was stirred vigorously for 10 minutes. The particles and supernatant were separated using external magnetic field. For the activation of amine-functionalized MNPs, 20 mL of 5 % glutaraldehyde solution was added and the mixture was shaken mechanically for 3 hours. The activated MNPs were separated using a magnet to remove unreacted glutaraldehyde. These MNPs were washed three times using the Coupling buffer and the supernatant was removed using magnet.

2.3.2. Avidin Coupling on the Activated Magnetic Nanoparticles

Five milligrams (5 mg) of streptavidin was dissolved in a 5 mL Coupling buffer. A volume of 100 μL of streptavidin was taken from the solution and was diluted by mixing it with 900 μL of phosphate buffered saline (PBS) solution (1:10 ratio). This solution was stored at 4ºC and was labeled as pre-coupling solution. Twenty-five milliliters (25 mL) of the streptavidin pre-coupling solution was prepared and added 5 mL of this mixture to each conical tube

with activated MNPs and was stirred mechanically for 24 hours. After mixing, the supernatant was separated using a magnet and was saved and labeled as the post-coupling solution. The pre-coupling solution and post-coupling solution were set-aside for the Coupling Efficiency Determination. The immobilized MNPs using streptavidin were coupled with biotinylated aptamers. The bovine serum albumin (BSA) was added before adding the human serum albumin. These albumin probed-aptamer MNPs were stored for the UPLC analysis. Further quantification of albumin in samples were executed using the Waters H.

2.4. Characterization of Iron (II, III) Oxide Magnetic Nanoparticles

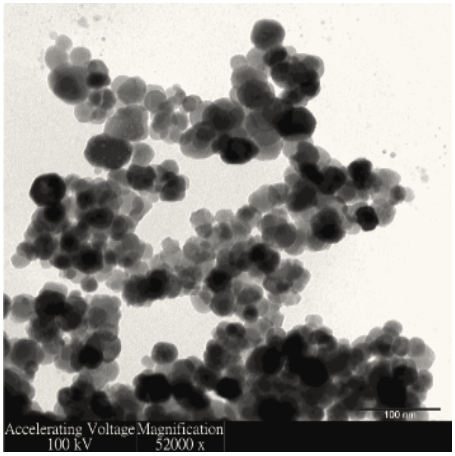
The functional groups present in the sample, Iron (II, III) oxide Magnetic Nanoparticles, were characterized using Fourier Transform Infrared Spectroscopy (FTIR, Shimadzu IR Prestige-21, UST). The IR spectra was recorded through the Diffuse Reflection Method and the DRS-8000 diffuse reflectance accessory was used. Surface study was made using the FEI Tecnai G2 20 Scanning TEM in University of Hong Kong -Electron Microscope. Thermogravimetric Analysis (TGA) of the bare MNPs and silane coated amine functionalized MNPs were carried out with a thermal analyzer Perkin Elmer TGA 400. The weights of the both samples were 20.961 mg. The analysis was performed from 50 ºC to 800 ºC at a heating rate of 10ºC/min.

3. Results and Discussion

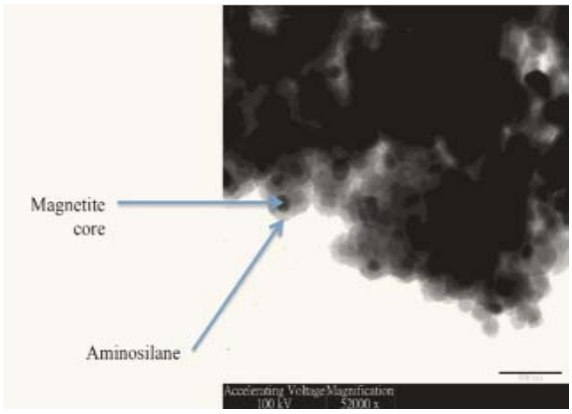
3.1. Characterization Study

The analysis of the FTIR spectroscopy confirmed the modification on the surface of the MNPs by the attachment of functional groups. Different volume ratios of the TEOS and APTES were used for the analysis. The FTIR bands at low wave numbers ($\leq$700 cm^{-1}) came from vibrations of Fe–O bonds of iron oxide [7]. The uncoated Fe_3O_4-MNPs had strong IR spectra at 570 cm^{-1} which indicates the characteristic bands of Fe_3O_4. This confirms that the MNPs used were Fe_3O_4. The absorption bands at 3300-3500 cm^{-1} corresponds to the OH stretching vibration due to physically absorbed water and the –OH groups on the surface of Fe_3O_4-MNPs. These spectra can be observed on both uncoated Fe_3O_4-MNPs and APTES-modified MNPs. The amine groups were attached to the surface of magnetic nanoparticles through condensation of APTES reaction. The Si-O stretching vibration observed at 1050 cm^{-1} confirmed the introduction of APTES on the surface of MNPs. This shows that the covalent bonds of Fe-O-Si are produced after the modification using APTES and TEOS. These bands cannot be observed on uncoated MNPs. Furthermore, the presence of the anchored propyl group attached to Si was confirmed by C-H stretching vibration observed at 2,850 cm^{-1} which are also absent in the spectra of the uncoated Fe_3O_4-MNPs. These results are evidences that APTES can be bonded on the surface bare MNPs through silanization reaction with –OH groups [28].

The size and morphology of the functionalized Fe_3O_4-MNPs MNPs were characterized by TEM. The TEM micrograph from the dried suspension of bare Fe_3O_4-MNPs is shown in Fig. 1.



(a)

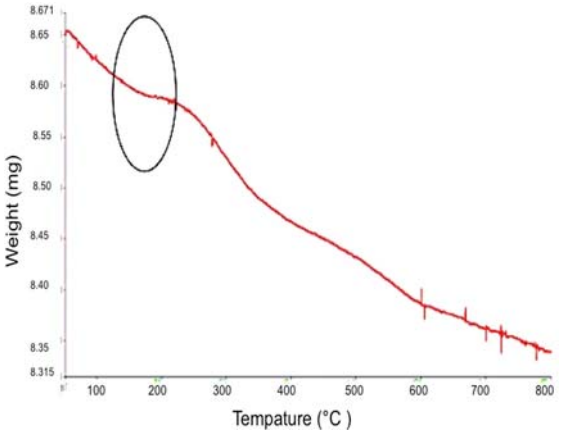


(b)

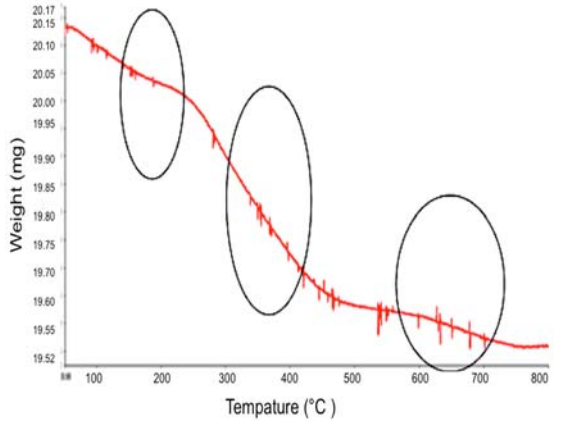
Fig. 1. (a) High Resolution TEM micrograph of bare Fe_3O_4-MNPs; (b) High Resolution TEM micrograph of functionalized MNPs.

The TEM micrograph of dried bare MNPs was used to calculate its particles size. The average diameter of the nanoparticles was approximately 33.33 nm. Magnetic nanoparticles with a size of less than 50 nm exhibits superparamagnetism. This proved that the MNPs used in the experiment had superparamagnetic properties. In addition, the nano-sized particles provides a large specific area that is needed for attaching functional groups (amine) on the surface of MNPs [25]. The TEM micrograph of the functionalized MNPs is shown in Fig. 1(b).

The indication of the coating formation on the surface of the Fe_3O_4-MNPs can be accomplished through the TGA measurement. The thermal stability of the bare Fe_3O_4-MNPs and APTES-modified MNPs were evaluated by TGA. In addition, thermal analysis further supports the existence of aminopropyl chain in the sample. Fig. 2(a) and Fig. 2(b) shows the graphs of the bare Fe_3O_4-MNPs and APTES-modified MNPs, respectively.



(a)



(b)

Fig. 2. (a) TG curve of bare Fe_3O_4-MNP; (b) TG curve of APTES-modified MNPs.

Upon heating a small amount of the sample, the Fe_3O_4-MNPs shows a small weight loss between 150 °C to 200 °C, mainly due to the loss of physically adsorbed water in both bare Fe_3O_4-MNPs and functionalized Fe_3O_4-MNPs. The weight loss at temperature between 300 °C to 400 °C is due to the loss of surface hydroxyl groups, which is also present in both bare Fe_3O_4-MNPs and functionalized Fe_3O_4-MNPs. Furthermore, a small weight loss of some organic moiety in the functionalized MNPs, which was absent in bare MNPs, were shown between 600 °C to 700 °C. This result shows that the APTES formed a coating on the surface of the MNPs and is stable up to 700 °C.

3.2. Coupling Efficiency of Streptavidin Uptake on the Functionalized MNPs

The quantitative adsorption of streptavidin on the surface of amine functionalized MNPs was determined in terms of its coupling efficiency. The % streptavidin uptake was obtained by measuring the absorbance of both pre-coupling and post-coupling solutions prepared at 280 nm. The equation below is used to calculate the % streptavidin uptake using diffident molar ratios of TEOS-APTES (1:2, 2:1 and 4:1):

$$\% \text{ Protein uptake} = \frac{\text{Precoupling Abs (A)-Post coupling Abs(A)}}{\text{Precoupling Abs (A)}}$$

The highest protein uptake computed (76.67 %) came from the TEOS: APTES sample with a ratio of 1:2.

3.3. Quantification of the Trace Albumin in Spiked Buffer Samples Using Ultra Performance Liquid Chromatography (UPLC)

The detection of albumin using spiked buffer samples was performed through UPLC which can analyze albumin with high resolution and sensitivity. Fig. 3 shows the chromatograph of the albumin liquid using HPLC.

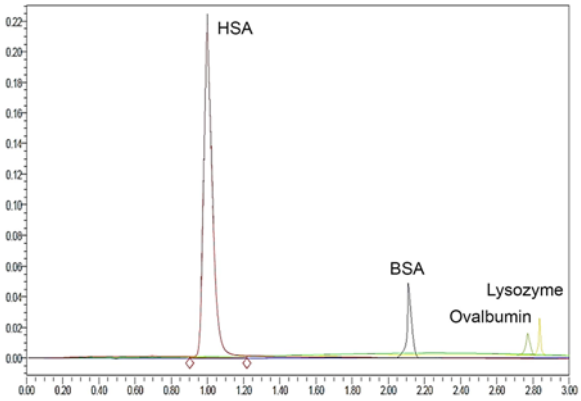


Fig. 3. Chromatograph of albumin using spiked buffer samples.

The concentration of albumin were 0.001, 0.01, 0.1, 1, 10, 1000 ppb were mixed with different proteins such as bovine serum albumin (BSA), ovalbumine, and lysozyme. It can be observed in the chromatograph on Fig. 3 that only human serum albumin (HSA) prominent peaks compared to the other proteins. This only means that the aptamer used in the study is highly selective to HSA. Table 1 shows the summary of the results obtained.

Fig. 4 shows the graph of the concentration of HSA versus the absorbance. The response of the HPLC in absorbance is directly proportional to the log of the

concentration of albumin with a linearity of 0.976. There is a noticeable change from 0.001 µg/mL to 10 µg/mL. A constant response was obtained upon reaching 100 to 300 µg/mL. The LOD of the UPLC analysis was 1.40 ppb.

Table 1. Test for specification on mixed solutions.

Protein	Conc. Standard (µg/mL)	Conc. in spiked buffer solution (µg/mL)	Peak Area	Repeata-bility %RSD, n=4
HSA	0.1	0.1	35679	3,61
BSA	0.1	0.1	3321	10.9
Lysozyme	0.025	0.025	612	21.35
Ovalbumin	0.05	0.05	301	18.35

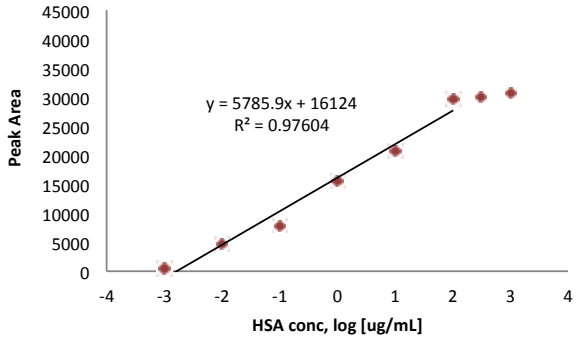


Fig. 4. Concentration of HSA versus the absorbance using UPLC.

The highest trace of Albumin in spiked buffer samples were detected using UPLC with an uptake of 55.03+1.15 % for a 0.01 ppm HSA concentration.

4. Conclusion

In this study, an effective method in functionalizing the surface of the iron (II, III) oxide or magnetite magnetic nanoparticles (Fe_3O_4-MNPs) was conducted. The Fe_3O_4-MNPs were functionalized by the application of silica coating and amine groups on the surface of the particles. These MNPs were activated by the glutaraldehyde for the attachment of streptavidin. The coupling efficiency of streptavidin using TEOS-APTES in 1:02 ratio was 76.67 %. The immobilization of aptamer on the surface of the amine functionalized MNPs was done and the HSA was successfully attached on the surface of the particles via avidin-biotin linkage. The functionalized MNPs were characterized using, FTIR, TEM and TGA analyses. For the advancement of this study, the use of a synthetic urine sample for the detection of microalbuminuria is recommended. The quantification of HSA in both buffered and synthetic urine samples shall be further examined using a Piezoelectric Quartz Cystal Sensor (PQC).

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