J. Iran. Chem. Soc., Vol. 8, No. 4, December 2011, pp. 944-950.

JOURNAL OF THE
Iranian
Chemical Society

Interaction of Bare and Gold-Coated Superparamagnetic Iron Oxide Nanoparticles with Fetal Bovine Serum

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(Received 21 December 2010, Accepted 18 February 2011)

Superparamagnetic iron oxide nanoparticles (SPIONs) are being increasingly used in various biomedical processes such as hyperthermia, cell and protein separation, enhancing resolution of magnetic resonance imaging and drug delivery. Here, SPIONs were prepared by optimized co-precipitation of iron chlorides in basic medium and then coated with gold. Bare SPIONs and Au-coated SPIONs were characterized by TEM before incubation with fetal bovine serum for 0.5, 1, 2, 4, 8 and 24 h. After these interaction times, the mixture was deposed on a small column in a strong magnetic field (MACS®system). The SPIONs were retained; different washing fractions were collected and studied by UV-Vis spectroscopy and by 1D gel electrophoresis. The study revealed the presence of proteins in the washing solutions and confirmed the strong interaction of the protein with the SPIONs.

Keywords: Interaction, Gold-coated iron oxide nanoparticles, Fetal bovine serum, UV-Vis spectroscopy, Gel electrophoresis

INTRODUCTION

The use of nanoparticles (NPs) is on the increase in many areas, especially in biomedicine for diagnostics and therapeutics [1-4]. NPs can interact with cells, tissues and all molecules present in the living organisms. NPs are commonly known to adsorb different proteins (referred to as "protein corona") [5,6] whose composition and affinity kinetics depend on the physicochemical properties of the NPs. Therefore, what the biological environment such as biological fluids, cells, tissues, and organs, actually "sees" while interacting with NPs, is completely different from the original surface properties of NPs. More specifically, NPs are taken into the intracellular medium through active, energy-dependent processes [4,7-11].

There are two kinds of coronas: soft and hard. The soft protein corona is a dynamic protein layer whose composition can be changed in time due to the exchange kinetics of the medium proteins. It is notable that the soft corona makes the primary contact with the cells [4,5,12,13], thus interfering with the *in vitro* cytotoxicity assessments of NPs (*e.g.* MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay) leading to a severe error in the obtained cytotoxicity data [14,15]. After the creation of hard corona (*i.e.* static layer, very slow exchange of proteins), the obtained cytotoxicity errors will be significantly reduced [14,15]. It should be noted that transferring the soft corona to the hard one requires some time which is related to the biophysiochemical properties of the formed bio-nano interface [16].

Little work on the interaction and the organization of the NPs has been carried out. However, when NPs interact with proteins, they can alter protein conformation, perturb their function and lead to toxicity [17]. Thus, it is very important to study and examine the behavior of NPs when they are in the presence of proteins and particularly to explore their

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hazardous effect on human health.

Protein structural changes after interaction with NPs have been mainly described by infrared, circular dichroism, fluorescence and other methods allowing the monitoring of the secondary structure of proteins [18,19]. Recently, Calzolai *et al*. [20] showed the possibility of identifying the Au-NP-protein interaction site at amino acid scale by NMR. Maiorano *et al*. [21] reported the effect of protein-NP complexes on the cellular response. They compared the impact of different common cellular media (DMEM and RPMI) and highlighted that the choice of the cellular medium is very important for the dynamic studies of protein-NP interactions.

In this work, both bare and gold-coated superparamagnetic iron oxide NPs (SPIONs) were synthesized and incubated with fetal bovine serum (FBS) in different period of time. SPIONs were characterized by TEM and the protein corona was studied by UV-Vis spectroscopy and 1D gel electrophoresis.

MATERIALS AND METHODS

Materials

Analytical grade of iron chloride salts (*i.e.* $FeCl_2$ and $FeCl_3$) and sodium hydroxide (NaOH) were purchased from Merck Inc. and were employed without further purification. Fetal bovine serum, which was used as the protein source, was obtained from Invitrogen. Gold salt ($HAuCl_4$), NH_2OH and poly-L-histidine, with molecular weight of ≥5,000, were purchased from Sigma-Aldrich. Other solvents were reagent grades and used without any further purification.

Synthesis of SPIONs

SPIONs were prepared according to the optimized co-precipitation process [22]. Briefly, the de-ionized (DI) water was de-oxygenated using bubbling of argon gas; iron salts, with a molar fraction of 2 (Fe^{3+}/Fe^{2+}), were dissolved in de-oxygenated DI water with HCl molarity of 1, respectively. The NPs were prepared using drop-wise addition of a predetermined mixture of iron salts to the base medium (*i.e.* NaOH) under an argon atmosphere.

Synthesis of Gold-Coated SPIONs

Gold was coated on the surface of SPIONs following a procedure published elsewhere [23]. Briefly, poly-L-histidine was added to the prepared SPIONs solution and the pH of the solution was adjusted to 5-6 using 0.1 N HCl; in this way, the polymer could be attached to the surface of the naked SPIONs via either physical or chemical binding. Poly-L-histidine was used as the templates for direct gold nucleation and growth on the surface of SPIONs.

After incubation for 60 min, the particles were collected by a strong magnet and redispersed in DI water. The procedure was repeated several times followed by the addition of $HAuCl_4$ (w/w 1%) to the NPs suspension. After the interaction time of 20 min and controlling the pH amount of 9-10 with NaOH, $NH_2OH.HCl$ was added and the mixture was stirred. Gold shell was formed on the surface of SPIONs. The suspension was washed several times and redispersed in DI water and stored at 4 °C.

Interaction of SPIONs with Protein

The SPIONs were incubated with FBS for periods of 0.5, 1, 2, 4, 8 and 24 h at temperature of 5 centigrade degree, respectively. In order to obtain a similar protein to the NP surface ratio, the ratio of protein solution to NP surface was fixed at 2.8 ml m^{-1} [2-6]. After the selected interaction time, the proteins with SPIONs were run through a strong magnetic field using magnetic-activated cell sorting (MACS®). Thus, the SPIONs were fixed inside the magnetic column and the flow-through fraction was collected. The fixed NPs were washed with a variety of washing solutions, including PBS, 0.01, 0.1, 0.5 and 1 M of KCl, respectively and stored. Finally, the column was removed from the magnetic field and NPs were fully removed and stored. Figure 1 shows schematically the experimental setup and the washing steps. All of the collected solutions, containing the interacted proteins, were analyzed by Bradford method and SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis; 15%) [18].

The NPs were characterized as follows. After placing and drying a drop of the colloidal suspension of SPIONs on a copper grid, the size and shape of the magnetic NPs were evaluated with a Phillips CM200 transmission electron microscope (TEM) equipped with an AMT 2 × 2 CCD camera at an accelerating voltage of 200 kV. In order to investigate the existence of proteins in the washing solutions, UV-Vis spectroscopy of the stored samples was performed on a Lambda 950 spectrophotometer (PerkinElmer, USA) from 300

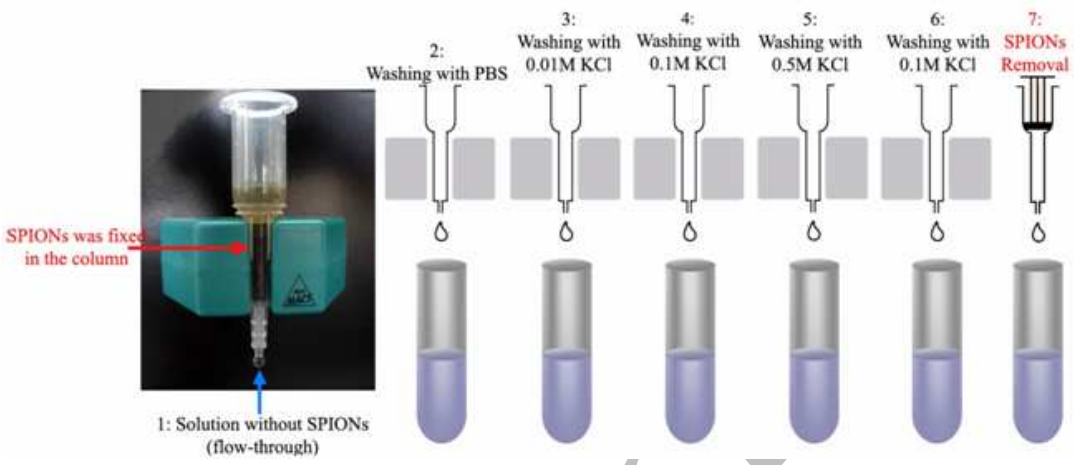

Fig. 1. Schematic representations of the magnetic separation method and employed washing steps.

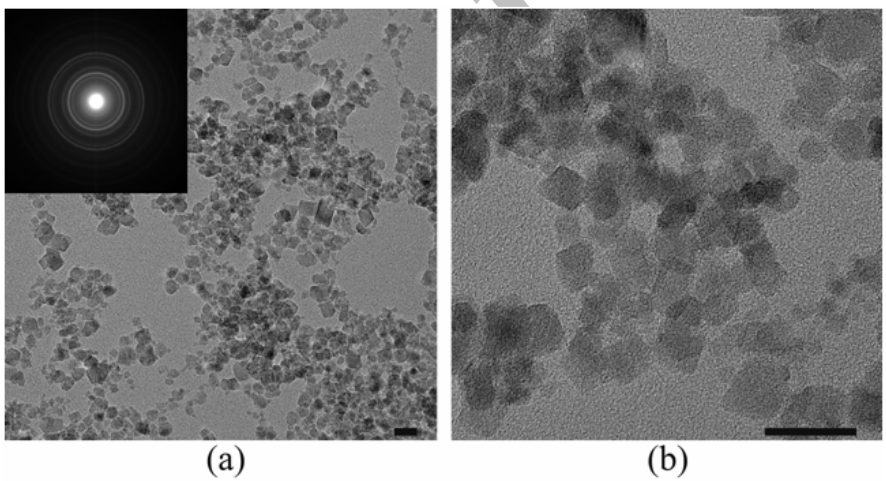

Fig. 2. (a) TEM image of synthesized SPIONs; inset at the top left is selected area diffraction pattern of the image; (b) TEM image of prepared SPIONs with higher magnification showing the narrow size distribution of obtained NPs; (scale bar is 20 nm).

to 800 nm wavelengths. Using the Bradford assay, which is based on the direct binding of Coomassie brilliant blue G-250 dye to proteins at arginine, tryptophan, tyrosine, histidine, and phenylalanine residues, the presence of the proteins could be monitored by probing the absorption peak shift at 595 nm. A more precise view of the presence of protein in the solutions together with their molecular weight was probed by SDS-PAGE (15%) following the procedure reported previously [24].

RESULTS AND DISCUSSIONS

In order to gain comprehensive information on the biophysicochemical properties of both as-synthesized SPIONs and the gold-coated ones, their size and distributions were carefully determined by TEM, and the formation of the protein corona was probed by UV-Vis absorption spectroscopy together with 1D gel electrophoresis. Figure 2 shows the information regarding morphology, size and size distribution

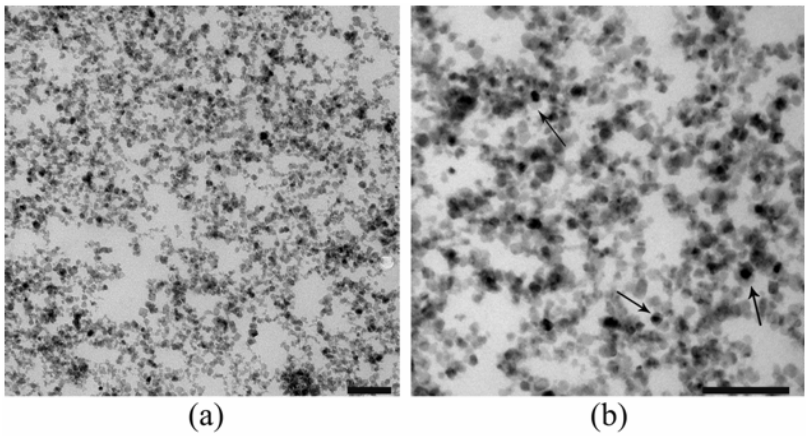

Fig. 3. (a) TEM image of gold-coated SPIONs; (b) TEM image of coated SPIONs with higher magnification showing either the formation of gold shell (see arrows for representation of some well-cleared coated NPs) or the narrow size distribution of obtained NPs; (scale bar is 50 nm).

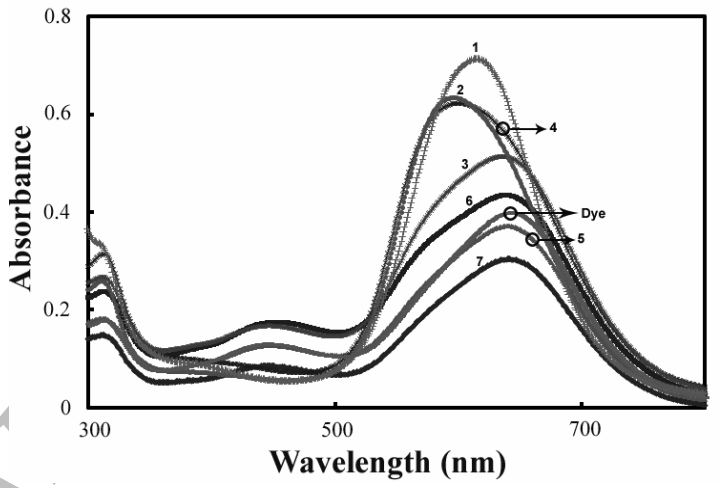

Fig. 4. UV-Vis spectroscopy of all washing solutions showing the absorbance changes associated with interactions of proteins with Coomassie brilliant blue G-250 dye in washing steps of 1, 2, and 4 (numbers in the figure showing the order of washing steps).

of the prepared SPIONs. The selected area diffraction pattern of the TEM images confirms the appropriate crystallinity of the obtained materials. In order to define the polydispersity of the thickness of the protein coating on the surface of SPIONs, statistical analysis was made [25]. The results confirmed the formation of SPIONs with spherical morphology with quite narrow size distribution (15 ± 2 nm), which is essential for obtaining reliable data on the formation of protein corona. TEM image of the gold-coated SPIONs also shows the formation of monodispersed coated particles (see Fig. 3).

The characterization of SPIONs interaction with FBS was performed using the Bradford assay. Figure 4 shows the UV-Vis absorption spectra and the shift of dye absorption maximum to the lower wavelength for the washing steps 1, 2

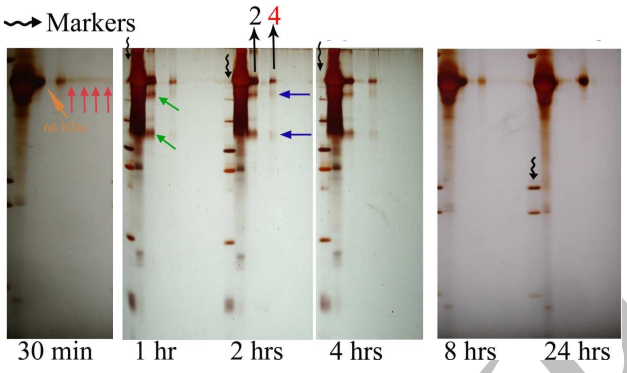

Fig. 5. SDS-PAGE of washing solutions for various protein-SPIONs incubation time.

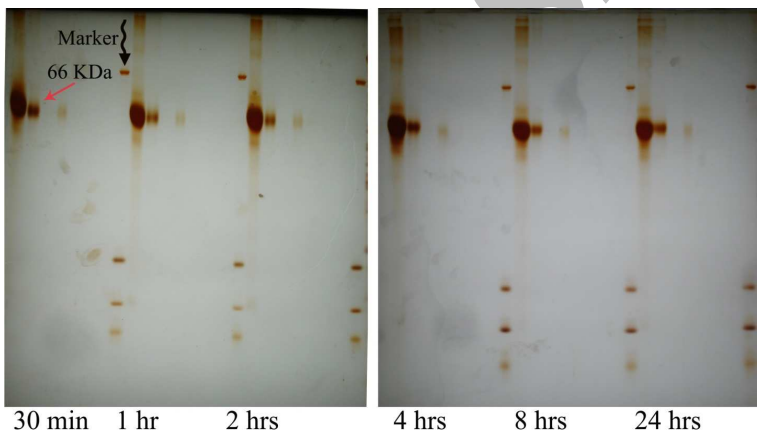

Fig. 6. SDS-PAGE of washing solutions for various protein and gold-coated SPIONs incubation time.

and 4. The peak-shifts confirm the presence of proteins in the washing solutions and the strong affinity of proteins to the surface of SPIONs. Similar results were obtained for the coated SPIONs (data not shown). In order to isolate and characterize the observed protein-SPIONs (both bare and the gold-coated one) complexes in washing stages, more specifically for steps 2 and 4 (see Fig. 1), both attachment affinity and time dependent monitoring of protein coronas were probed using SDS-PAGE. A number of methods have been developed for the investigation of protein corona on the surface of various NPs [21]. Among these, centrifugation route has been recognized to provide reliable information on the gradient of the absorbed proteins on the surface of NPs [6]. However, the centrifugation method suffers from the detachment of loosely-attached proteins [26]. Therefore, the distinguishing feature of our setup together with the magnetic properties of SPIONs is that there is no removal of loosely-attached proteins and the precise gradient of protein coronas could be obtained. All washing solutions (see Fig. 1 for details) at various times were analyzed by SDS-PAGE to probe the gradient of the attached proteins to the surface of SPIONs. Figure 5 shows the obtained gels of all solutions for the selected times. The results confirm the rich protein profiles for stages 1 of the solution (*i.e.* the flow-through fraction), 2 (*i.e.* washed with PBS), and 4 (*i.e.* washed with 0.1 M KCl). As seen in Fig. 4, there are significant differences between the protein profiles at various times of interactions, confirming the variation of proteins compositions and densities by interaction

time. After the 30 min. interaction of SPIONs with FBS, all washing solutions contained considerable amounts of high molecular weight proteins (*i.e.* molecular weight of 66 KDa) such as albumin (see red arrows in Fig. 4). Since the protein bands remained even after being washed with all the solutions (see the last red arrow which is responsible for NPs after washing steps), it can be concluded that these proteins do attach to the surface of SPIONs with strong affinity. By increasing the protein-NP interaction time to 1 h, the bands for stages 5-7 entirely vanished confirming the reduction in the binding affinity of high molecular weight proteins; in contrast, additional bands had disappeared in stages 2 and 4, showing that the lower molecular weight proteins had made their entry into the protein corona (see green and blue arrows in Fig. 4). Similar results were obtained by further increasing the protein-NP interaction time up to 4 h. The trace of low molecular weight proteins disappeared at the protein-SPIONs interaction time of 8 h and the same results were obtained for the protein-SPIONs interaction time of 24 h indicating the formation of hard corona by proteins with high molecular weights.

Figure 6 shows the SDS-PAGE results of gold-coated SPIONs. There are no considerable differences between the gels confirming that the composition of protein corona did not change during the different incubation times. Furthermore, there is no trace of competition between the lower molecular proteins and the higher one (*i.e.* 66 KDa) due to the existence of highly compatible and neutral surface of the coated SPIONs.

CONCLUSIONS

SPIONs were obtained by coprecipitation of ferric and ferrous ions in basic medium. Gold-coated SPIONs were prepared as described. Both kinds of SPIONs were incubated with FBS at different periods of time (between 0.5 and 24 h). After magnetic filtration on MACS®, the different fractions were studied by UV-Vis spectroscopy and by 1D gel electrophoresis. With respect to bare SPIONs, following the incubation time, the results showed the formation of a hard corona by proteins with more or less high molecular weights. As for Au-coated SPIONs, the incubation time had no influence on the composition of protein corona.

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