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Preparation of anti-human cardiac troponin I immunomagnetic nanoparticles and biological activity assays

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Abstract

Maghemite nanoparticles (MNPs) were synthesized by chemical coprecipitation and coated with *meso*-2,3-dimercaptosuccinic acid (HOOC-CH(SH)-CH(SH)-COOH or DMSA). The morphology and properties of the nanoparticles were characterized by TEM, XRD, Zeta Potential Analyzer and VSM. Subsequentially, the anti-human cardiac troponin I (cTnI) immunomagnetic nanoparticles (IMNPs) were prepared by grafting anti-human cTnI antibodies on the surface of DMSA-coated MNPs using the linker of EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride). The conjugation amount of the antibodies and the activity of IMNPs was evaluated by enzyme linked immunosorbent assay (ELISA) and Western blotting. The results show that the physical and chemical adsorption occurred at the same time, but the former was unstable and apt to desorb, and the maximum conjugation amount of antibody was about 96 μg on the 0.1 mg MNPs by covalent bond. The stability was also investigated, and after 300 days the antibodies on the IMNPs remained the biological activity.

Keywords: Maghemite; Immunomagnetic nanoparticles; Activity assays; Antibody; Cardiac troponin I

1. Introduction

Over the past two decades, maghemite (γ-Fe_2O_3) or magnetite (Fe_3O_4) nanoparticles have shown great attractive applications including cellular labeling and separation [1], specific tissue repair [2], drug delivery [3], magnetic resonance imaging [4], hyperthermia [5], and magnetofection [6], etc. Recently more and more researchers have fixed attention on the separation, detection and target capability of maghemite nanoparticles (MNPs) in earlier diagnosis and treatment for some disease because of their stability and biocompatibility. When conjugated with biomolecules such as antibodies, biotin and enzyme and so on, the MNPs could form lock-and-key interactions with target molecules of antigen, streptavidin and substrate, respectively [7,8]. Therefore, adequate amount of biomolecules and biological activity on the surface of MNPs is the key of their specific recognition and target capability.

Traditional methods of conjugation antibody include simple physiochemical adsorption, Langmuir–Blodgett method and covalent conjugation [9]. The first two methods are more susceptible to desorption due to the reversible nature of non-covalent conjugation. Therefore, many scientists have been employing the last method to graft antibodies on the magnetic nanoparticles and recognize the antigen [10], cell [4], virus [11], etc. It is of significance to investigate the conjugation efficiency, conjugation activity and the conjugation stability of antibodies on the surface of MNPs. Some researchers employed thermogravimetry, chemical analysis, fluorescence spectroscopy and chemisorption isotherm analysis to evaluate conjugation efficiency [7,12]. But, to the best of our knowledge, the conjugation activity and stability have not been investigated.

Cardiac troponin I (cTnI) is a subunit of cardiac troponin complex. Following the myocardial damage, the troponin complex is broken up and the individual protein components are released into the bloodstream [13]. The cTnI has high tissue specificity

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which ensures an accurate assay [14–16]. Therefore, cTnI in the serum of patients has been considered as the "gold standard" for diagnosis of myocardial injury [17–19]. It is of significance to enrich and detect low-abundance cTnI sensitively for diagnosis of myocardial damage. MNPs are attractive tool for rapid detection and quantification the target biomolecules. The cTnI can be captured by the specific anti-human cTnI antibody conjugated with MNPs (immunomagnetic nanoparticles, IMNPs), and then separated and enriched by a gradient magnetic field.

Here we focused on the preparation of the IMNPs, the conjugation amount of antibodies, the biological activity and the stability of the IMNPs by biological method of the enzyme linked immunosorbent assay (ELISA) and Western blotting. For the first time the morphology and the stability of IMNPs was offered.

2. Materials and methods

2.1. Synthesis of DMSA-coated MNPs

Fe_3O_4 nanoparticles were synthesized by chemical coprecipitation of Molday [20]. Typically, a solution of mixture of $FeCl_3$ and $FeSO_4$ (molar ratio 2:1) was prepared under N_2 protecting, then, enough ammonia aqueous solution was dropped into it with violently stirring. After 30 min, the resulting Fe_3O_4 nanoparticles were washed immediately with distilled water for five times by magnetic separation. The final magnetite nanoparticles were dispersed in distilled water with concentration of 0.128 M and pH 3.0, and oxidized into more stable MNPs (γ-Fe_2O_3) by air at the temperature of 90 °C.

According to the process described in literature [21,22], MNPs were coated with *meso*-2,3-dimercaptosuccinic acid (DMSA). One finally obtained an aqueous sol of MNPs, which was stable in a large pH range (from 3 to 11), in suitable ionic strength and in various buffers [23]. At the same time, the unbound carboxyl groups (-COOH) and thiol groups (-SH) could be used to covalently graft biological ligands. Therefore, DMSA-coated MNPs were desired carrier in biological applications. In the work, the carboxyl groups were used to graft biomolecules.

2.2. Characterization of DMSA-coated MNPs

The particle size and morphology of the samples was determined by transmission electronic microscopy (TEM, JEOL, JEM-200EX). Powder X-ray diffraction (XRD, Rigaku, D/Max-RA, $\lambda = 1.5405 \times 10^{-10}$ m, CuK) was used to determine the crystal structure of the samples. Surface charge measurements were performed with a Zeta Potential Analyzer (BECKMAN, Delsa 440SX). The magnetic measurements were carried out with a Vibrating Sample Magnetometer (VSM, Lakeshore 7407).

2.3. Preparation of IMNPs

Here EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) (Pierce Company) was used to activate terminal carboxyl groups on the MNPs for the conjugation with anti-human cTnI monoclonal antibodies. cTnI from human myocardium muscle and the monoclonal anti-human cTnI antibodies used in the experiment were obtained from Research Institute of Cardiovascular Disease of First Affiliated hospital of Nanjing Medical University. The five eppendorf tubes of DMSA-coated MNPs suspension (1 mL per tube at 0.1 mg/mL) were washed three times with distilled water using NdFeB magnet to sediment and then resuspended in 1 mL PBS buffer (0.1 M, pH 5.6). Fresh EDC solution was added to the tubes at ambient temperature with gently shaking for 5 min before 96, 192, 288, 384 and 480 μg antibody solution being added and mixed gently, respectively, at 4 °C for 4 h. The MNPs conjugated with antibodies (IMNPs) were separated and washed three times for removing superfluous antibodies using magnetic decantation and then resuspended in 1 mL PBS solution (0.1 M, pH 7.0).

2.4. Biological activity assays

2.4.1. Enzyme linked immunosorbent assay (ELISA)

Activity assays of IMNPs were carried out by an indirect ELISA method. IMNPs solution was diluted 100 times with PBST (0.1 M, 0.05% (v:v) Tween 20) buffer and added into the wells (100 μL/well) of the 96-well microplate which was coated previously with cTnI. The microplate was incubated for 1 h at 37 °C in the water bath. After removing the solution, the microplate was rinsed with Tris buffer (0.1 M Tris, pH 8.0, 0.1 M NaCl) three times. The goat anti-mouse IgG labeled peroxidase (Sigma) diluted 2000 times with PBST solution containing 0.05% BSA was added (100 μL/well) and incubated 30 min at 37 °C. The microwells were then rinsed with Tris buffer five times and drained. Developed by addition of substrates, namely 3,3′,5,5′-tetramethybenzidine peroxidase (TMB-H_2O_2), for 10 min at 37 °C, the reaction was stopped by 2 M H_2SO_4 (50 μL/well). The optical density (OD) at 450/630 nm was read immediately in an Ultra Microplate Reader (ELX808 IU, BIOTEK). All samples were repeated three times and the average results were calculated.

2.4.2. Western blotting activity assays

The cTnI solution (10 μg per lane) and low molecule weight marker (Sigma) were separated with Tricine-SDS-PAGE gel, and then transferred to Immun-Blot polyvinylidene difluoride (PVDF) membrane at 30 mA constant current overnight. Following transfer, the marker was stained with coomassie brilliant blue R250 solution, the other membrane was rinsed with TBS, the blank binding sites were blocked with TBS solution containing 1% bovine serum albumin (BSA) overnight at 4 °C. The membrane was then incubated with 2.5 μg IMNPs in TBS solution for 2 h at 37 °C, and 2.5 μg antibodies as contrast sample. Incubated membranes were subsequently washed three times with TBST (TBS containing 1% (v:v) Tween-20) for 5 min per time under constant shaking. Secondary antibody of goat anti-mouse IgG alkaline phosphatase antibodies (Sigma) was diluted with TBS solution containing 1% BSA and incubated with the membranes for 1 h at 37 °C. Then the membranes were washed three times with TBST and then TBS solution for 5 min per

time, respectively. Finally, the membranes were stained with 5-bromo-4-chloro-3-indoxyl phosphate (BCIP)/nitro-blue tetrazolium (NBT).

3. Results and discussion

The MNPs were synthesized by chemical coprecipitation and stable MNPs suspension was obtained via surface coating with DMSA. The morphology of particles was observed by TEM, as shown in Fig. 1, most of the particles are quasi-spherical and with an average diameter of 18 nm. The distribution of particles diameter is shown in Fig. 2.

The phase identification of the MNPs was performed by XRD (as seen in Table 1). The *d*-spacing obtained experimentally accords with the theory values, which indicate that the sample is an inverse cubic spinel structure.

Table 1
XRD data for the DMSA-coated MNPs

	1	2	3	4	5	6	7
d_{xRD}	4.70	2.93	2.51	2.08	1.70	1.60	1.47
d_{theory}	4.82	2.95	2.51	2.09	1.70	1.61	1.47
Crystalline plane (*h1k*)	111	220	311	400	422	511	440

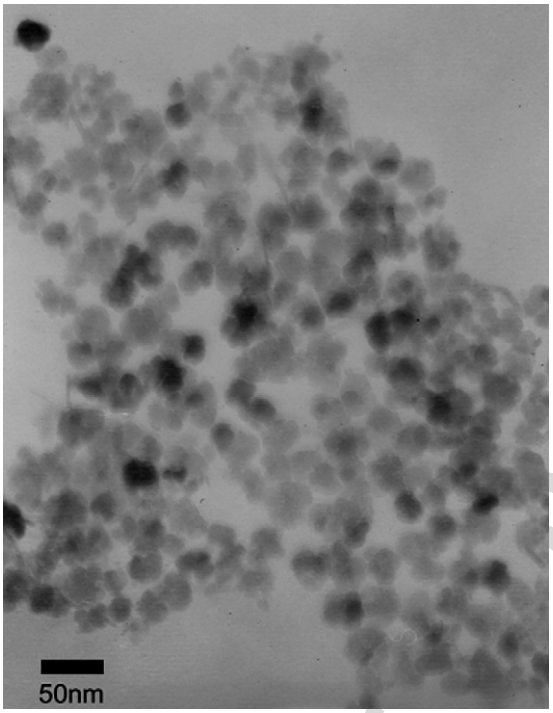

Fig. 1. TEM photograph of the DMSA-coated MNPs.

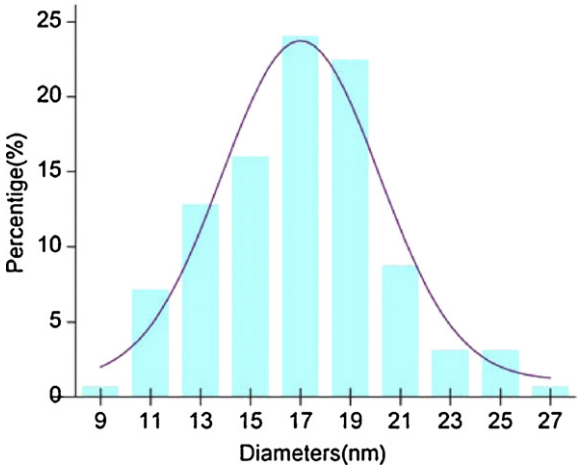

Fig. 2. Diameter distribution of the DMSA-coated MNPs.

The magnetic property of the MNPs was measured by VSM. Fig. 3 shows the magnetization as a function of an external field at ambient temperature. The magnetic parameters such as saturation magnetization (Ms) and coercivity (Hc) are given in the figure. Note that the value of Ms, 67.6 emu/g at ambient temperature, is slightly smaller than that of bulk maghemite (75.0 emu/g), which reflects the nanocrystalline nature of the particles. The difference in the saturation magnetization between the bulk and the nanosized material can be attributed to the small particle size effect [24].

Zeta (ξ) potential measurement of MNPs as a function of pH has been performed to confirm the surface charge property. The pH values of the MNPs suspension were adjusted to 3, 5, 7, 9, 11, respectively. The results indicate that the particles have high negative potential at the pH range mentioned above, resulting in the strong electrostatic repulsion to protect the particles from congregating. On the other hand, ξ potentials increase with pH values, especially in low pH range as shown in Fig. 4. This is attributed to the ionization of -COOH and -SH groups with increasing pH values.

EDC is a carboxyl and amine-reactive cross-linker, which reacts with a carboxyl group first and forms an amine-reactive *O*-acylisourea intermediate that quickly reacts with an amino group to form an amide bond and release of an isourea by-product. In the experiment, the free carboxyl groups of DMSA chelated on the surface of MNPs were activated by EDC solution, and then immediately formed the amide bonds with the free amino groups of the antibody. The covalent conjugation scheme of the IMNPs

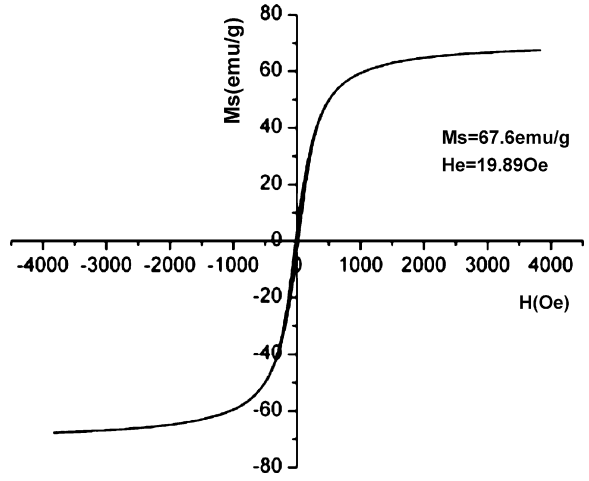

Fig. 3. Magnetization vs. applied magnetic field for the DMSA-coated MNPs.

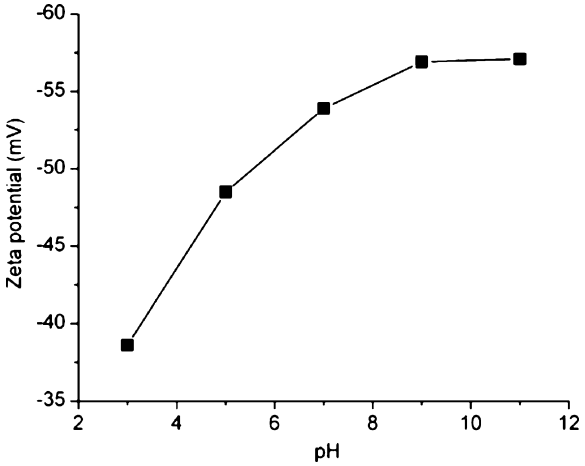

Fig. 4. pH-dependent zeta potential curves of the DMSA-coated MNPs.

is shown in Fig. 5 according to the instruction of EDC offered by Pierce Company.

The morphology of IMNPs was observed by TEM. The IMNPs suspension was dropped on a carbon-coated 300-mesh copper grid and dried under ambient conditions. To directly and clearly observe the antibodies on the surface of IMNPs, the copper grid was negatively stained by phosphotungstic acid and then dried in the air. As shown in Fig. 6, a visible and white layer of antibodies can be observed around the particles because of the lower electron density of antibodies compared with γ-Fe_2O_3 [12]. This provides direct evidence for that the antibodies are successfully grafted to the surface of MNPs. To the best of our knowledge, this is for the first time that the morphology of IMNPs has been obtained.

To demonstrate the activity of the IMNPs, cTnI was used as target molecules for detection by an indirect ELISA method. Considering the possible effect of MNPs on OD values and the non-specific adsorption of IMNPs, the DMSA-coated MNPs, the boiled IMNPs for 5 min and the IMNPs were also investigated contrastively. As indicated in Fig. 7, the antibodies on the IMNPs remain their biological activity (see the cyan column). The DMSA-coated MNPs and the boiled IMNPs have scarcely any effect on the OD values compared with the IMNPs (see the green and blue columns, respectively). This suggests that the IMNPs have non-specific adsorption.

The maximum conjugation amount of antibodies on the 0.1 mg MNPs was obtained for the fresh IMNPs. As demonstrated in Fig. 8, the immunoactivity strengthens with increasing the quality of antibodies up to about 288 μg, after that, the

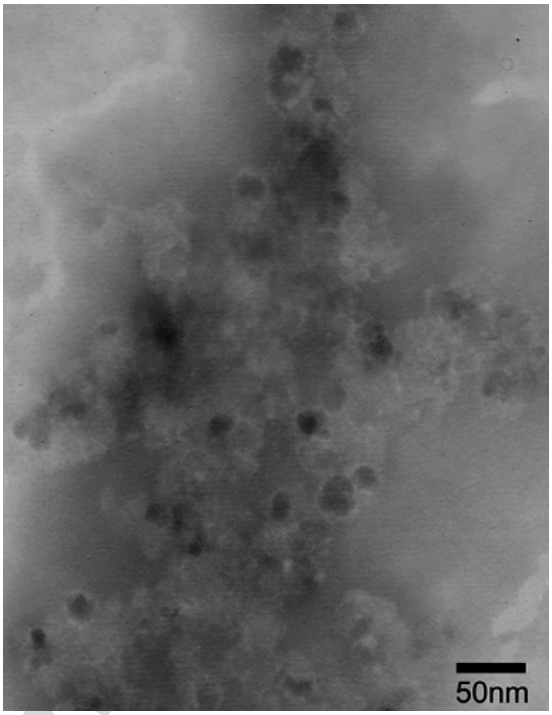

Fig. 6. TEM photograph of anti-human cTnI IMNPs.

OD value remains constantly. This means that the nanoparticles are completely covered by the antibodies, and the more antibodies could not be conjugated. The maximum conjugation amount of antibody on the nanoparticles used in the experiment is 288 μg with maximum immunoactivity, but the physical adsorption probably occured.

The stability of the IMNPs was investigated, which was necessary for application in practice. All the tubes of samples were washed again by magnetic decantation and resuspended in 1 mL PBS storage solution when stored for 3 days. The biological activity of resuspension was also measured by ELISA method. After 300 days, all the resuspension was treated and measured in the same way. The results (shown in Fig. 9) demonstrate that the biological activity of the IMNPs with 96 μg antibody remains constantly, however, the other four samples' activity weakened with time. Interestingly, all samples showed nearly consistent activity after 300 days. We infer that approximate 96 μg antibodies are adequate to cover the surface of 0.1 mg nanoparticles, while superfluous antibodies covered on the nanoparticles by physical adsorption. Desorption took place after storing for a period of time, resulting in the activity weakening of the some

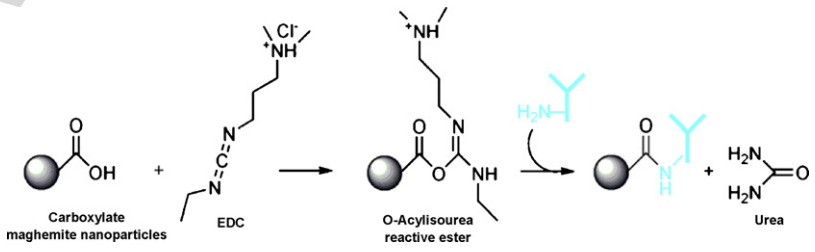

Fig. 5. The conjugation scheme of the anti-human cTnI antibodies and DMSA-coated MNPs.

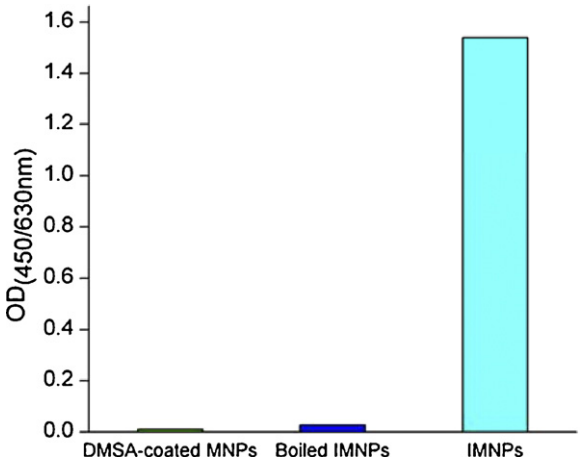

Fig. 7. The effect of the non-specific adsorption of the DMSA-coated MNPs and the boiled IMNPs on OD values, and the activity of the IMNPs.

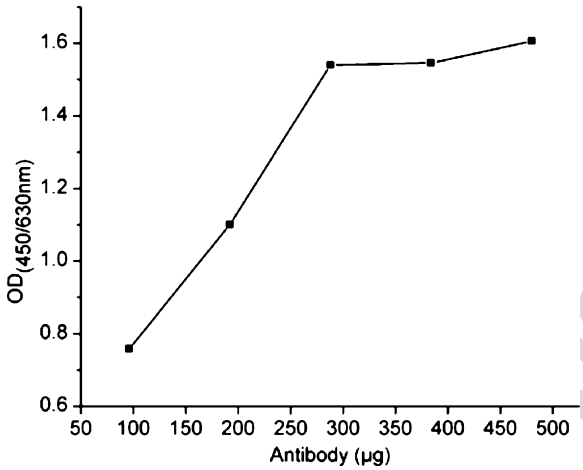

Fig. 8. The activity curve of the IMNPs with different amount of anti-human cTnI antibodies.

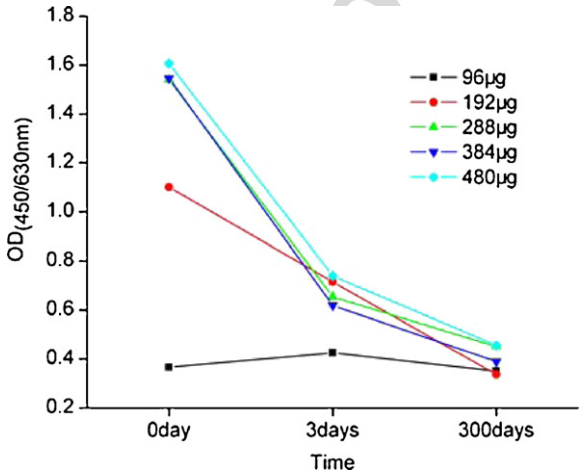

Fig. 9. The stability curves of the IMNPs with different amount of anti-human cTnI antibodies.

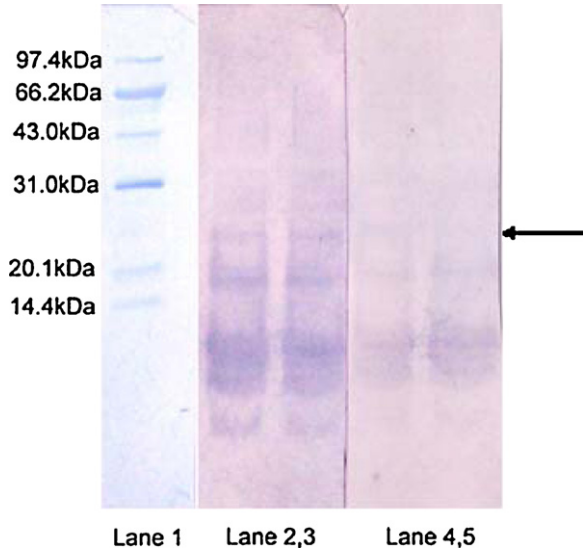

Fig. 10. The results of western blotting on PVDF membrane. (Lane 1) The low molecule weight marker stained with coomassie brilliant blue; (lanes 2 and 3) the anti-human cTnI antibody stained with BCIP/NBT, (lanes 3 and 4) the anti-human cTnI IMNPs stained with BCIP/NBT, the band pointed by the arrow refers to the position of cTnI (24 kD).

samples, while the antibody molecules conjugated by chemical adsorption remained their affinity. In brief, the maximum conjugation amount of the antibodies with covalent bond could be considered roughly as about 96 μg.

An additional important method, Western blotting, was carried out to confirm the affinity of the cTnI and the IMNPs. Compared with ELISA, Western blotting has more specific affinity and sensitivity. As shown in Fig. 10, the PVDF membranes display the bands of cTnI at 24 kDa (pointed by the arrow), where the cTnI is successfully recognized by the IMNPs. Other bands appear because of the partial decomposition of cTnI. Similar results were obtained in repeated experiments independently.

4. Conclusions

DMSA-coated MNPs were synthesized by chemical coprecipitation and characterized by TEM, XRD, Zeta Potential Analyzer and VSM. The anti-human cTnI IMNPs were prepared by grafting antibodies on the surface of DMSA-coated MNPs using EDC cross-linker. The biological methods of ELISA and Western blotting were employed to evaluate the biological activity of IMNPs. The results show that the antibodies remain their specific affinity and biological stability after 300 days. The maximum conjugation amount of antibodies on the 0.1 mg MNPs is about 96 μg by covalent bonds. Especially, for the first time the morphology and the stability of IMNPs was investigated.

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