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Ultra-small particles of iron oxide as peroxidase for immunohistochemical detection

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Abstract
Dimercaptosuccinic acid (DMSA) modified ultra-small particles of iron oxide (USPIO) were synthesized through a two-step process. The first step: oleic acid (OA) capped Fe_3O_4 (OA-USPIO) were synthesized by a novel oxidation coprecipitation method in H_2O/DMSO mixing system, where DMSO acts as an oxidant simultaneously. The second step: OA was replaced by DMSA to obtain water-soluble nanoparticles. The as-synthesized nanoparticles were characterized by TEM, FTIR, TGA, VSM, DLS, EDS and UV–vis. Hydrodynamic sizes and Peroxidase-like catalytic activity of the nanoparticles were investigated. The hydrodynamic sizes of the nanoparticles (around 24.4 nm) were well suited to developing stable nanoprobes for bio-detection. The kinetic studies were performed to quantitatively evaluate the catalytic ability of the peroxidase-like nanoparticles. The calculated kinetic parameters indicated that the DMSA-USPIO possesses high catalytic activity. Based on the high activity, immunohistochemical experiments were established: using low-cost nanoparticles as the enzyme instead of expensive HRP, Nimotuzumab was conjugated onto the surface of the nanoparticles to construct a kind of ultra-small nanoprobe which was employed to detect epidermal growth factor receptor (EGFR) over-expressed on the membrane of esophageal cancer cell. The proper sizes of the probes and the result of membranous immunohistochemical staining suggest that the probes can be served as a useful diagnostic reagent for bio-detection.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Magnetic nanoparticles (Fe_3O_4) are widely used in various fields such as magnetic resonance imaging (MRI), diagnostics, magnetic cell separation and hyperthermia [1–7]. Recently, it was also reported that Fe_3O_4 nanoparticles possess intrinsic peroxidase-like activity. The mechanism of the peroxidase-like activity may follow the Fenton reaction [8]. It can be written as (1) and (2), where k_1 and k_2 are the reaction rate constants.

$$Fe^{2+} + H_2O_2 \xrightarrow{k_1} Fe^{3+} + \cdot OH + OH^- \quad (1)$$

$$Fe^{3+} + H_2O_2 \xrightarrow{k_2} Fe^{2+} + \cdot OOH + H^+. \quad (2)$$

The hydroxyl radical (·OH) thus formed plays an important role in oxidation of enzyme substrates, such as 3,3′,5,5′-tetramethylbenzidine (TMB).

The peroxidase-like activity could be used in various applications, including those dependent on the detection of hydrogen peroxide. Peroxidase catalyzes oxidation of certain substrates to produce characteristic color in the presence of hydrogen peroxide. Such chromogenic reactions have become a useful tool in detection of hydrogen peroxide. For example, they were used for glucose detection [9], detection of hydrogen peroxide in acid rain and highly efficient catalytic oxidation of phenols [10, 11].

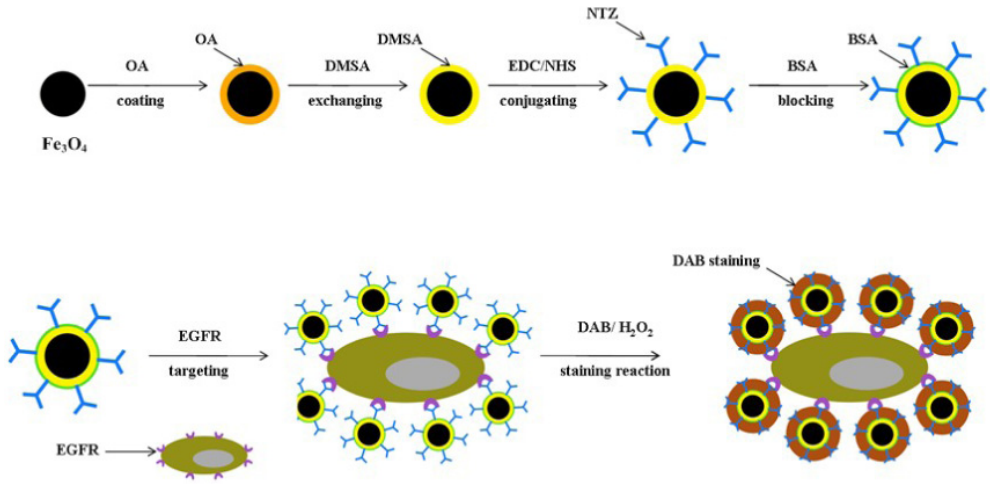

Figure 1. Synthetic procedure for the Ab-DMSA-USPIO probes and their biological application in immunohistochemical staining.

Immunohistochemical experiments are also based on the chromogenic reaction [12]. We can confirm two questions with the chromogenic reaction. (a) Did the Ab-DMSA-USPIO probes bind to the EGFR? (b) Did Fe_3O_4 nanoparticles possess intrinsic peroxidase-like activity? EGFR is a 170 kDa transmembrane tyrosine kinase receptor belonging to the ErbB family [13]. It is present in most epithelial tissues and plays an important role in cell growth and function. In addition, it can be inhibited by anti-EGFR mAbs. As for anti-EGFR mAbs, Nimotuzumab (also known as h-R3) is a humanized monoclonal antibody that recognizes the EGFR external domain with intermediate affinity (Kd $= 10^{-8}$) [14]. In preclinical *in vitro* as well as *in vivo* studies, Nimotuzumab demonstrated a remarkable anti-proliferative, pro-apoptotic and anti-angiogenic effect [15–17]. In this experiment, the esophageal cancer cell will show membranous immunohistochemical staining when the Ab-DMSA-USPIO probes bind to the EGFR.

Compared with naturally peroxidase enzymes (HPR), Fe_3O_4 nanoparticles are very stable and possess a rarely changed catalytic activity over a wide range of temperatures and pH values. In addition, Fe_3O_4 nanoparticles, much cheaper than HRP, can be easily synthesized via chemical means [18]. Currently, there are SPIO and USPIO on sale and they are modified by dextran or its derivatives. SPIO coated with dextran and its derivatives possess a large hydrodynamic size, and are also very expensive [19–21].

In our experiment, we used the small molecules Dimercaptosuccinic acid (DMSA) instead of the dextran and its derivatives. Here, we designed our probes as shown in figure 1: (a) the USPIO was modified with DMSA by a simple two-step process. (b) The Ab-DMSA-USPIO probes were synthesized via the method of bio-coupling reaction. The amino groups of Nimotuzumab were connected with the carboxyl groups of DMSA-USPIO under the effect of the cross-linking agent (EDC–NHS). (c) The Ab-DMSA-USPIO probes were used for immunohistochemical experiments.

2. Experimental section

2.1. Chemicals and reagents

All chemicals used in this experiment were of analytical reagent grade. Deionized water was used throughout the study. Ferrous sulfate ($FeSO_4{\cdot}7H_2O$), oleic acid, dimethyl sulfoxide (DMSO), n-hexane, acetone, glacial acetic acid, anhydrous sodium acetate, absolute alcohol, 2,3-dimercaptosuccinic acid and hydrogen peroxide (30%) were purchased from Sinopharm Chemical Reagent Co. Ltd. Dimethylbenzene, dipotassium hydrogen phosphate, sodium phosphate dibasic, sodium chloride, sodium borate and boric acid were reagents from Shanghai Lingfeng Chemical Reagent Co. Ltd. Tetramethylammonium hydroxide was supplied by Shanghai Zhuorui Chemical Reagent Co. Ltd. Albumin bovine (BSA) was obtained from Nanjing Bookman Biotechnology Ltd. Diaminobenzidine (DAB), pepsin, hematoxylin, HRP-anti-IgG and positive control slides (overexpressed EGFR) were produced by Fuzhou MaiXin biotechnology development Co. Ltd. Nimotuzu monoclonal antibody was served by Beijing BaiTai biopharmaceutical Co. Ltd.

2.2. Preparation of oleic acid (OA) modified USPIO

$FeSO_4{\cdot}7H_2O$ (3 mmol), oleic acid (10 ml), 25% (w/w) N $(CH_3)_4OH$ (10 ml) and DMSO (35 ml) were mixed in a 100 ml three-necked bottle. Under the protection of N_2, the mixture was magnetically stirred and refluxed at 140 °C for 1 h. At last, the black precipitate was obtained via magnetic separation and was washed three times with absolute ethyl alcohol. The final sample (OA-USPIO) was obtained by vacuum drying.

2.3. Preparation of dimercaptosuccinic acid (DMSA) modified USPIO

The OA-USPIO (45 mg) was dissolved in n-hexane (9 ml) and DMSA (45 mg) was dissolved in acetone (9 ml). They

were mixed in a 50 ml three-necked bottle and the mixture was mechanically stirred at 60 °C for 4 h. The black precipitate was obtained via magnetic separation and washed with absolute ethyl alcohol three times, it was then dispersed in water. After that, it was poured into a dialysis bag (retention molecular weight is 8000d) in water for three days and centrifuged at 5000 rpm for 40 min and 10 000 rpm for 50 min to remove any agglomerates. The final sample (DMSA-USPIO) was filtrated in a 0.22 μm membrane.

2.4. Preparation of Ab-DMSA-USPIO probes

Borate buffer (PH = 9.0, 1 ml), DMSA-USPIO (14.9 mmol l^{-1}, 100 μl), EDC (10 μg μl^{-1}, 50 μl) and NHS (10 μg μl^{-1}, 70 μl) were added into a black cap bottle in an orderly way and the mixture was shaken at 25 °C for 30 min. The bio-coupling reaction was started by adding Nimotuzumab (250 μg) while continually shaking at 25 °C for 2 h. The blocking reaction was carried out by adding 4% BSA solution (400 μl) with continual shaking at 25 °C for 1 h. After that, it was purified by gel chromatography (GE sephacryl s-300). The final sample was stored at 4 °C for immunohistochemical experiments.

2.5. Peroxidase-like activity of DMSA-USPIO and Ab-DMSA-USPIO probes

The DMSA-USPIO and Ab-DMSA-USPIO probes were diluted in various concentrations of Fe_3O_4 (1.274, 2.41, 3.43, 4.35, 4.955 and 5.693 $\times$ 10^{-7} M) in a reaction system with 200 μl of reaction buffer (0.2 M NaAc, pH = 3.6), 10 μl 30% H_2O_2 and 5 μl TMB solution (10 mg ml^{-1}, dissolved in DMSO). This experiment was performed by the BIO-RAD model 680 microplate reader. The reaction times of the DMSA-USPIO and Ab-DMSA-USPIO probes with TMB and H_2O_2 were 7 min and 17 min respectively.

2.6. Steady state kinetic analysis of DMSA-USPIO

The steady state kinetic assays were carried out at room temperature in a reaction system with 5 μl of DMSA-USPIO (1.25 μg μl^{-1}) in 200 μl of reaction buffer (0.2 M NaAc, pH = 3.6) in the presence of H_2O_2 and TMB. The kinetic analysis of the DMSA-USPIO with TMB as the substrate was performed by adding 10 μl 30% H_2O_2 and different amounts (0, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 μl) of TMB solution (10 mg ml^{-1}, dissolved in DMSO). The kinetic analysis of the DMSA-USPIO with H_2O_2 as the substrate was performed by adding 5 μl TMB and different amounts (0, 2, 10, 20, 40, 60, 80, 100 μl) of 30% H_2O_2 solution. All the reactions were monitored in timescale mode at 650 nm using the BIO-RAD model 680 microplate reader. Catalytic parameters were determined by fitting the absorbance data to the Michaelis–Menten equation. The Michaelis–Menten equation, written as (3), describes the relationship between the rates of substrate conversion by an enzyme and the concentration of the substrate.

$$V = \frac{V_{\max} \times [S]}{K_m + [S]}. \tag{3}$$

In this equation, V is the rate of conversion, $V_{\max}$ is the maximum rate of conversion, $[S]$ is the substrate concentration, and K_m is the Michaelis constant. The Michaelis constant is equivalent to the substrate concentration at which the rate of conversion is half of $V_{\max}$ and K_m approximates the affinity of the enzyme for the substrate.

2.7. Immunohistochemical experiments of Ab-DMSA-USPIO probes

Immunohistochemistry experiments were performed to detect EGFR over-expressed on the membrane of esophageal cancer cells using DAB as a chromogenic substrate. DAB (3,3′-diaminobenzidine) is a commonly used chromogen for immunohistochemical staining. In the presence of H_2O_2, DAB is converted to an insoluble brown reaction product and water by the enzyme HRP (horseradish peroxidase). The reaction is something like this:

$$\mathrm{DAB} + \mathrm{H_2O_2} \xrightarrow{\mathrm{HRP}} \mathrm{DABppt} + \mathrm{H_2O}. \tag{4}$$

Ab-DMSA-USPIO probes possess intrinsic peroxidase-like activity. Combined with HRP-anti-IgG, they were used in this experiment. DAB staining was used to reveal the presence of probes. An experiment group and three control groups were set up to indicate that the probes can be served as an effective detection method of EGFR. The positive control slides were first submerged in dimethylbenzene three times, each for 10 min, and then in 100% ethanol twice, each for 10 min. They were submerged in different levels of alcohol (90%–70%), each for 10 min and then placed in distilled water. They were put in a wet box after they were fetched out and the water around the tissues on the slides was wiped off. 3% H_2O_2 was added to the tissues and the slides were kept in incubation for 15 min in darkness. Then distilled water was used to wash the slides, which again were submerged in PBS buffer (PH = 7.4) three times, each for 5 min. The fluids around the tissues on the slides were wiped off when they were fetched out to put into the box. 100 μl of pepsin was added on the slides which were kept in incubation of 37 °C for 30 min. Then, the slides were washed by PBS twice, each for 3 min. Afterwards, the experiment group was incubated with the probes (100 μl) at 37 °C for 30 min, the control-1 was incubated with PBS (100 μl) at 37 °C for 30 min, the control-2 was incubated with NTZ (100 μl) first at 37 °C for 30 min followed by incubation of the probes (100 μl) at 37 °C for 30 min, the control-3 was incubated with IgG (100 μl) first at 37 °C for 30 min and then incubation of HRP-Anti-IgG (100 μl) at 37 °C for 30 min. All of them were treated with DAB (100 μl) for 15 min. After being washed with PBS, they were counterstained with hematoxylin, then the slides were dehydrated through alcohols (70%–100%), each for 10 min. And then, the slides were submerged in dimethylbenzene three times, each for 10 min and sealed with balsam neutral. Finally, the slides were observed with a light microscope (AXIOVERT200, ZEISS).

The particle size and morphology of the USPIO obtained above were characterized by transmission electronic microscopy (TEM, JEOL JEM-2100). Fourier transform

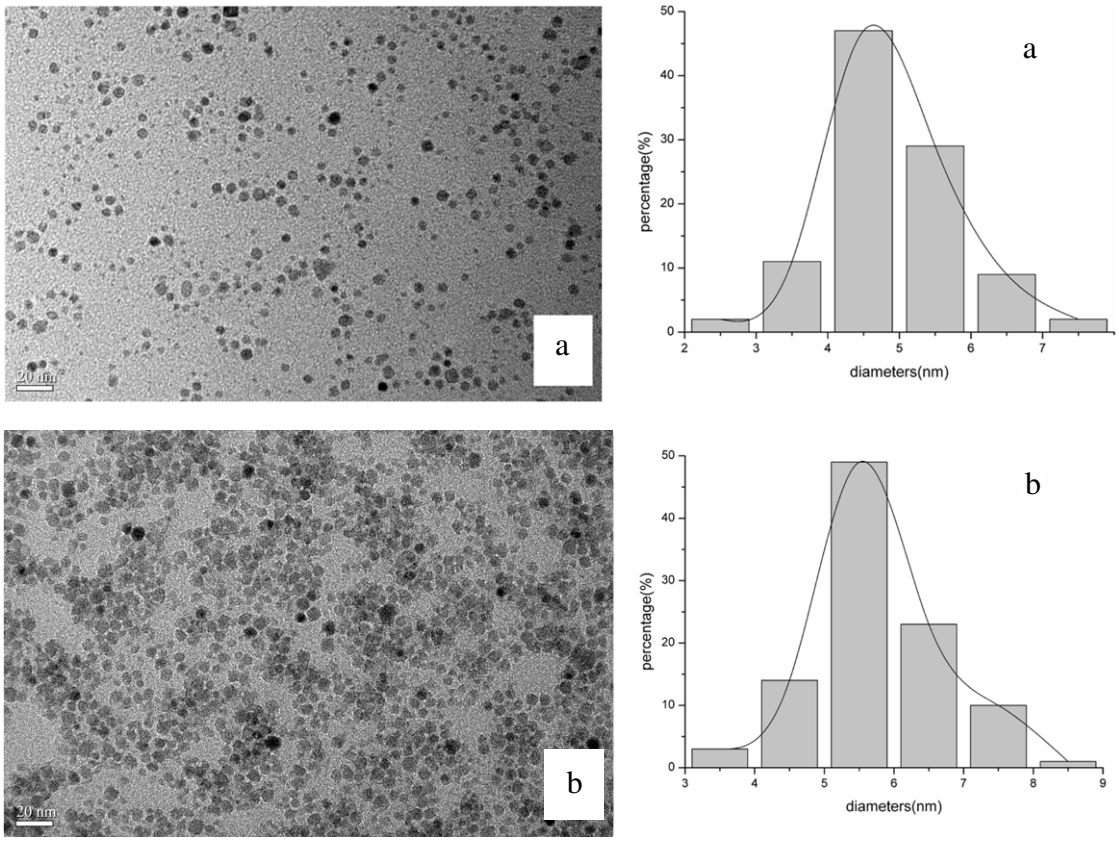

Figure 2. TEM photographs of (a) the OA-USPIO, (b) the DMSA-USPIO.

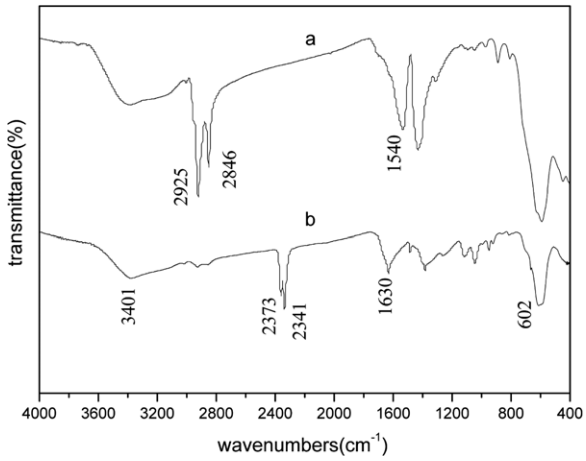

Figure 3. FTIR spectra of (a) the OA-USPIO, (b) the DMSA-USPIO.

infrared spectroscopy (FTIR) analysis was performed by an infrared spectrometer (Nicolet AVATAR 360 FTIR). Thermal analysis was measured by thermogravimetric analyzer (TGA, PerKinElmer DTA7). Magnetic measurements were carried out with a Lakeshore 7407 vibrating sample magnetometer (VSM). Hydrodynamic sizes were detected by Zeta potential laser particle size Analyzer (ZetaMaster3000, Marven). An energy dispersive spectrometer (EDS) was used to determine the element ratio of iron and sulfur. Ultraviolet visible (UV–vis) absorption spectra were measured on an UV–vis–NIR spectrophotometer (Shimadzu UV-3600, Japan).

2.8. Results and discussion

2.8.1. Characterization of the OA-USPIO and the DMSA-USPIO. TEM images (figure 2) show the mean diameter of the Fe_3O_4 core and corresponding distributions of the as-synthesized samples. For OA-USPIO, it possesses an average diameter 4.65 nm. After replacement by the DMSA, the particles tend to be slightly larger and the mean diameter is about 5.56 nm. These values are obtained from the measurement of about 100 particles.

The FTIR absorption spectra (figure 3) give a comparison between the OA-USPIO and DMSA-USPIO. In the spectrum of the OA-USPIO, the two peaks at 2846 and 2925 cm^{-1} are attributed to the C–H stretching vibration. The two peaks at 1540 and 1450 cm^{-1}, attributed to the COO^- stretching, reveal the existence of OA. In the spectrum of the DMSA-USPIO, the two peaks at 2341 and 2373 cm^{-1} are attributed to the S–H stretching vibration. The two peaks at 1630 and 1505 cm^{-1}, attributed to the COO^- stretching indicate the presence of DMSA [22]. The characteristic absorption peak of Fe–O is observed at 602 cm^{-1} for both of the OA-USPIO and DMSA-USPIO. In addition, the absorption bands near 3401 cm^{-1} are referred to the O–H stretching, indicating the presence of water remaining in the samples.

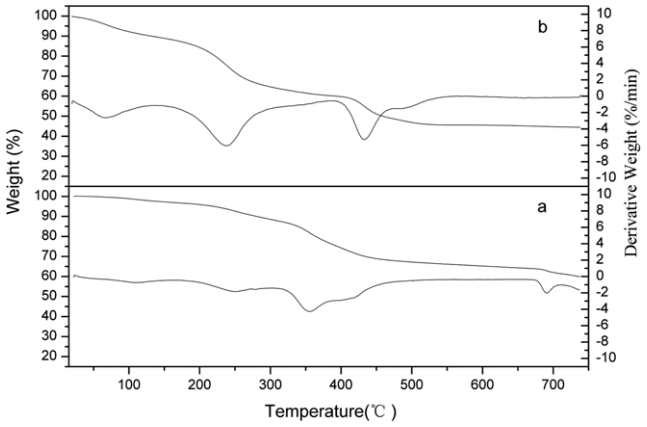

Figure 4. The thermal analysis curves of (a) the OA-USPIO, (b) the DMSA-USPIO.

2.8.2. Thermal analysis. The thermal analysis curves of the OA-USPIO and the DMSA-USPIO (figure 4) show that decomposition takes place in 25–750 °C temperature range. As for OA-USPIO shown by the curve (a) in figure 4, weight loss from 20 °C to 120 °C could be attributed to the evaporation of physically adsorbed water. Weight loss from 120 °C to 300 °C resulted from the degradation of chemically bound water while the weight loss from 300 °C to 750 °C resulted from removal of oleic acid molecules from the sample surface. As for DMSA-USPIO shown by the curve (b) in figure 4, apart from the physically adsorbed loss, weight loss from 120 °C to 400 °C was due to the decomposition of chemically bound water, which may be caused by higher hydrophilicity of DMSA. Weight loss from 400 °C to 750 °C resulted from removal of DMSA molecules from the powder surface.

From the curve without the impact of water, it can be calculated that the percentage of OA on the surface of the USPIO was about 31.8% and the percentage of DMSA on the surface of the USPIO was about 24.2%. Assuming one DMSA molecule exchanged with one oleic acid molecule, the weight loss of DMSA removed from the DMSA-USPIO should be 20.5% (near to the 24.2%). So there is approximately a monolayer of DMSA molecules on the USPIO.

2.8.3. Magnetism. The hysteresis loops of the OA-USPIO and the DMSA-USPIO (figure 5) were measured at room temperature with a vibrating sample magnetometer (VSM). Both of the samples possess superparamagnetic behavior. The saturation magnetization values (M_s) for the OA-USPIO and the DMSA-USPIO are 34.47 and 34.21 emu g^{-1}, respectively.

Furthermore, the oleic acid and the DMSA that coated the surface of the USPIO may have a negative effect on the saturation magnetization. The M_s of the Fe_3O_4 core in the OA-USPIO and the DMSA-USPIO were calculated to be 50.54 and 45.13 emu g^{-1}, respectively (table 1).

2.9. Coverage area of one DMSA

Since we know the mean diameter of the Fe_3O_4 core in DMSA-USPIO is 5.56 nm, the number of Fe atoms in one USPIO can

Table 1. The M_s for Fe_3O_4 cores based on TGA and VSM results.

Sample	Percentage of Fe_3O_4 core (%) (determined by TGA)	Total M_s (emu g^{-1}; determined by VSM)	The M_s of Fe_3O_4 core (emu g^{-1})
OA-USPIO	68.2	34.47	50.54
DMSA-USPIO	75.8	34.21	45.13

be calculated by means of following formula,

$$N_{Fe} = \frac{\frac{4}{3}\pi r^3 \rho_{Fe_3O_4} \times 3}{M_{Fe_3O_4}} \times N_A = 3636 \qquad (5)$$

where $M_{Fe_3O_4}$ refers to the molar mass of the USPIO, r is the mean radius of USPIO, $\rho_{Fe_3O_4}$ is the density of the USPIO, N_A is Avogadro's constant.

The atomic ratio of Fe/S ($N_{Fe}/N_s = 4$) was indicated by SEM–EDS elemental analysis. The number of DMSA molecules on the surface of every USPIO can be obtained as $3636/(4\times2) = 455$. As the USPIO is coated by approximately a monolayer of DMSA molecules, the coverage area of one DMSA can be calculated by means of following formula,

$$S_{DMSA} = \frac{S_{Fe_3O_4}}{N_{DMSA}} = \frac{4\pi r^2}{N_{DMSA}} = 0.21\ \text{nm}^2 \qquad (6)$$

where $S_{Fe_3O_4}$ refers to the surface area of USPIO and N_{DMSA} is the number of DMSA molecules on the surface of one USPIO.

2.9.1. The evaluation of Ab-DMSA-USPIO probes. Hydrodynamic sizes of the BSA, the DMSA-USPIO and the Ab-DMSA-USPIO probes blocked by BSA are shown in figure 6. The coupling reaction between Nimotuzumab and the DMSA-USPIO was first investigated. It can be seen in figure 6(b) that, before the bio-coupling, the DMSA-USPIO has a hydrodynamic size about 24.4 nm. After the reaction, the hydrodynamic size of the Ab-DMSA-USPIO probes (figure 6(c)) increases to around 35.8 nm. This is close to the simple addition of the dimensions from the DMSA-USPIO (24.4 nm) and the Nimotuzumab. There is a peak of 5.6 nm present in figure 6(c) due to the free BSA. The reasonable variation in the hydrodynamic size of the probes potently suggests that Nimotuzumab is effectively coupled to the DMSA-USPIO.

The UV–vis absorption spectrum (figure 7) shows the curves of the DMSA-USPIO, the Ab-DMSA-USPIO probes unblocked by BSA and the Ab-DMSA-USPIO probes blocked by BSA. In the case of uncoupled DMSA-USPIO (figure 7(a)), no obvious absorption peak could be found, while for Nimotuzumab coupled the DMSA-USPIO with no free Nimotuzumab (figure 7(b)), there is a slight absorption peak at 280 nm, which is due to the coupling of Nimotuzumab. For the Ab-DMSA-USPIO probes blocked by BSA with no free Nimotuzumab and BSA (figure 7(c)), there is an obvious absorption peak at 280 nm, which is due to the coupling of Nimotuzumab and the BSA. It is clear that, the Nimotuzumab were successfully coupled onto the DMSA-USPIO.

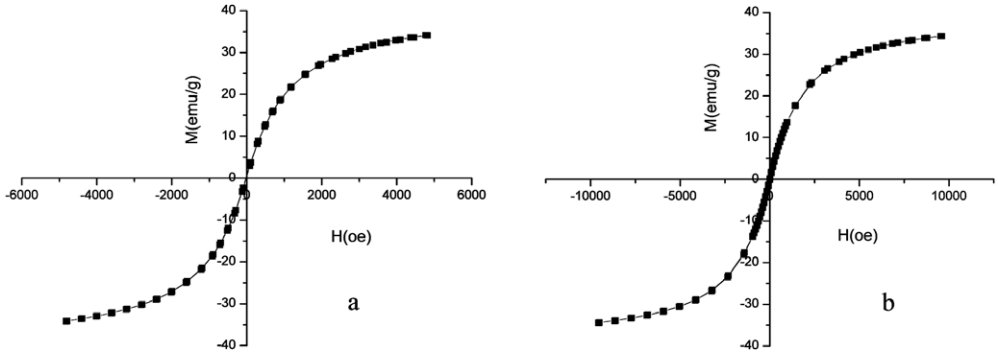

Figure 5. Magnetization curves of (a) the OA-USPIO, (b) the DMSA-USPIO.

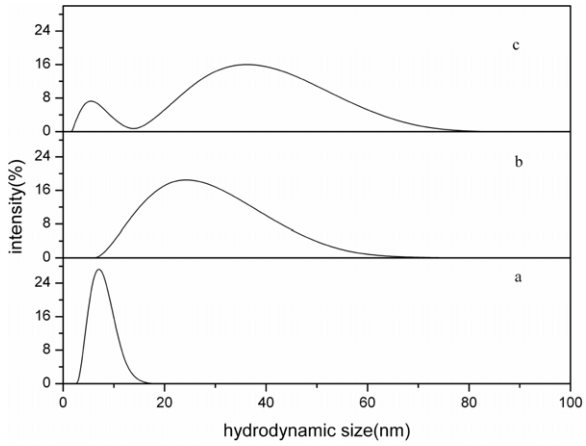

Figure 6. Hydrodynamic sizes of (a) BSA, (b) the DMSA-USPIO, (c) the Ab-DMSA-USPIO probes blocked by BSA.

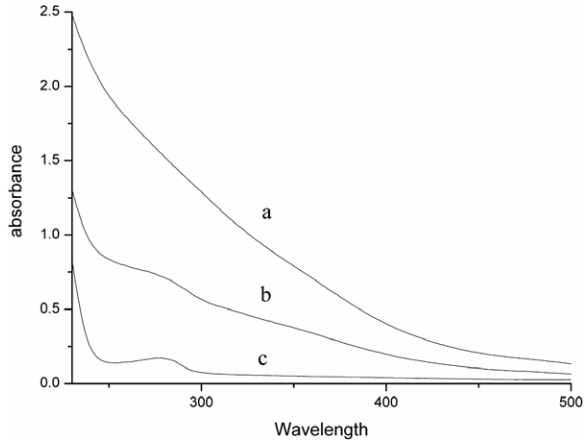

Figure 7. UV–vis absorption spectra of (a) the DMSA-USPIO, (b) the Ab-DMSA-USPIO probes unblocked by BSA, (c) the Ab-DMSA-USPIO probes blocked by BSA.

The peroxidase-like behavior of the synthesized DMSA-USPIO and Ab-DMSA-USPIO probes was indicated using TMB as chromogenic substrate. TMB has been proved to be a noncarcinogenic derivative and could be oxidized into a blue color reaction product with absorbance at 650 nm in the presence of H_2O_2 [23, 24].

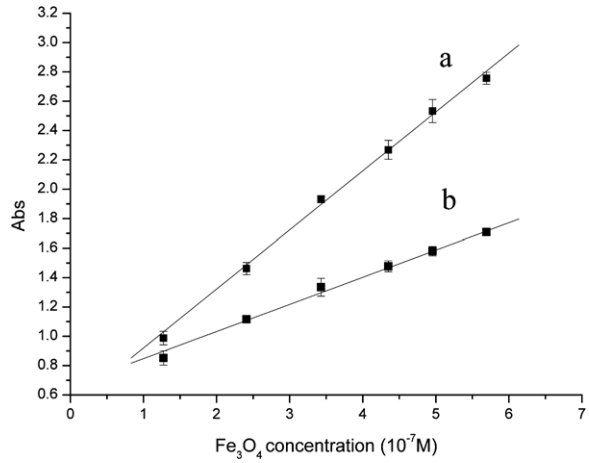

Figure 8. Peroxidase-like activity of (a) the DMSA-USPIO (b) the Ab-DMSA-USPIO probes.

Figure 8 shows the Fe_3O_4 concentration dependency of the peroxidase-like activity. There is an absorbance linear increase with a correlation coefficient of 0.9977 as a function of the Fe_3O_4 concentration (figure 8(a)), indicating that DMSA-USPIO can be used as a mimic enzyme label in bioanalysis. The Ab-DMSA-USPIO probes also have this change. There is an absorbance linear increase with a correlation coefficient of 0.9985 as a function of the Fe_3O_4 concentration (figure 8(b)). It can be confirmed that both of the DMSA-USPIO and the Ab-DMSA-USPIO probes possess peroxidase-like activity. DMSA-USPIO could be taken as a kind of enzyme to displace the expensive HRP and the Ab-DMSA-USPIO probes were applied in the immunohistochemistry experiments to further confirm the potential application in bio-detection.

2.9.2. Steady state kinetics. The steady state kinetic parameters were determined at room temperature and the DMSA-USPIO was performed as the catalytic agent. Absorbance data were back-calculated to concentration by the Beer–Lambert law using a molar absorption coefficient of 39 000 M^{-1} cm^{-1} for TMB-derived oxidation products. Steady state reaction rates at different concentrations of substrate were obtained by calculating the slopes of the initial absorbance changes with time. Figure 9 indicates the reaction catalyzed by DMSA-USPIO and displayed in Michaelis–Menten kinetics.

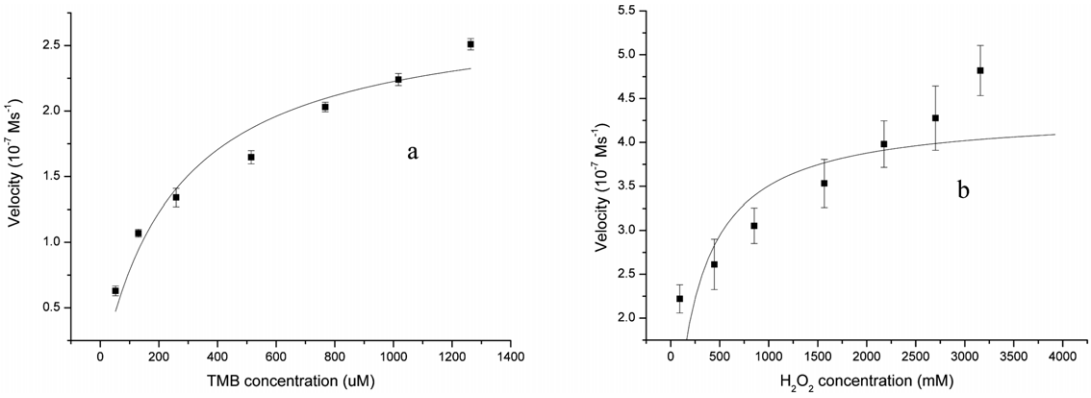

Figure 9. Michaelis constant of DMSA-USPIO (a) with TMB as the substrate, (b) with H_2O_2 as the substrate.

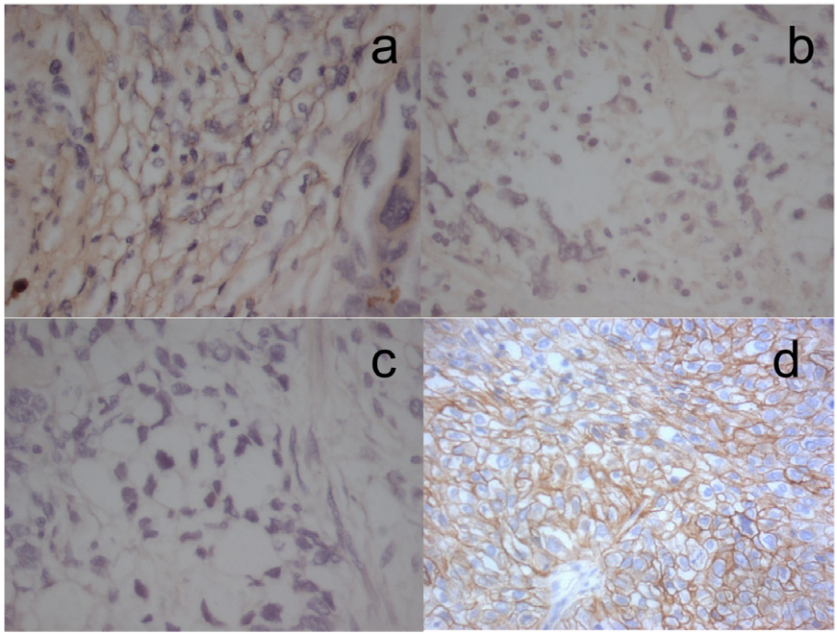

Figure 10. Immunohistochemistry staining of DAB (a) the Ab-DMSA-USPIO probes, (b) control-1, (c) control-2, (d) control-3.

Table 2. Kinetic parameters of DMSA-USPIO and HRP. $[E]$ is the enzyme (or Fe_3O_4) concentration, K_m is the Michaelis constant, V_{max} is the maximal reaction velocity and k_{cat} is the catalytic constant, where $k_{cat} = V_{max}/[E]$.

Sample	$[E]$ (M)	Substrate	K_m (mM)	V_{max} (m s^{-1})	k_{cat} (s^{-1})
DMSA-USPIO	2.79×10^{-7}	TMB	0.255	2.794×10^{-7}	1
DMSA-USPIO	2.79×10^{-7}	H_2O_2	237	4.336×10^{-7}	1.554
HRP	2.5×10^{-11}	TMB	0.434	10.00×10^{-8}	4.00×10^{3}
HRP	2.5×10^{-11}	H_2O_2	3.7	8.71×10^{-8}	3.48×10^{3}

Data was fitted to the Michaelis–Menten equation to obtain the catalytic parameters, as shown in table 2. The K_m value of DMSA-USPIO with H_2O_2 as the substrate was significantly higher than that for HRP, consistent with the observation that a higher concentration of H_2O_2 was required to get maximal reaction velocity for the DMSA-USPIO. The K_m value of the DMSA-USPIO with TMB as the substrate was a little less than that of HRP, suggesting that the DMSA-USPIO has a slightly higher affinity for TMB than HRP. At the same molar concentration, the kcat value of DMSA-USPIO and HRP showed a similar level of activity. The k_{cat}/K_m value of DMSA-USPIO and HRP with TMB as substrate appeared similar. The kcat/Km value of DMSA-USPIO with H_2O_2 as substrate was significantly smaller than that for HRP, corresponding to the larger Km value for H_2O_2. As a catalytic agent, compared with HRP, the DMSA-USPIO can exhibit similar catalytic efficiency. There may be many ferrous ions at the surface of DMSA-USPIO, providing more active sites for catalysis, while HRP has only one active site [25].

2.9.3. Immunohistochemistry analysis. Figure 10 shows the DAB staining of immunohistochemistry experiments.

From the four pictures, there is obvious membranous immunohistochemical staining in figures 10(a) and (d). But in control-1 (figure 10(b)) and control-2 (figure 10(c)), we can find that there are no obvious changes, which shows that EGFR were detected on the membrane of esophageal cancer cell with the probes. It reveals that the DMSA-USPIO possesses a capability of simulating peroxidase and the probes can be considered to be an effective diagnostic reagent.

3. Conclusion

As we know, the DMSA-USPIO possesses the capability of simulating peroxidase. It can play an important role in catalytic reaction. In our work, the Ab-DMSA-USPIO probes were designed and synthesized. The results have shown that the intrinsic peroxidase-like activity of DMSA-USPIO and the specificity of the Nimotuzumab were combined. The probes were successfully utilized to detect the EGFR over-expressed on the membrane of esophageal cancer cell. Therefore, it can be used for immunohistochemical detection *in vitro*. Moreover, the hydrodynamic size of the Ab-DMSA-USPIO probes was controlled around 35.8 nm. The ultra-small hydrodynamic size helps the probes avoid the RES clean and they have a long half-life in the blood circulation. Conceivably, it can also serve as a medical diagnosis regent and medicament for oncotherapy *in vivo*.

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