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Aqueous nickel-nitrilotriacetate modified $\text{Fe}_3\text{O}_4\text{-NH}_3^+$ nanoparticles for protein purification and cell targeting

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Abstract

A comprehensive totally aqueous phase synthesis of nickel-nitrilotriacetate (Ni-NTA) modified superparamagnetic Fe_3O_4 nanoparticles is presented. The $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$ nanoparticles are able to perform efficient and specific purification of 6-His tagged proteins from crude cell lysates, as evidenced by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. The average binding capacity, as demonstrated by streptopain (M_w 42 kDa), is 0.23 mg/mg (protein/ $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$). Considering the high affinity and specificity of the binding between hexahistidine motif and Ni-NTA, Ni-NTA modified nanoparticles could act as a module to carry 6-His tagged proteins on the particle surface with molecular orientation control, since only the 6-His domain could be attached. These modularly designed functional nanoparticles enhance cancer cell targeting, as supported by the *in vitro* receptor mediated targeting assay using RGD-4C-6-His fusion peptide. The nanoparticles show no significant hemolysis for human blood and could be investigated further for their *in vivo* functional imaging applications.

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

1. Introduction

Nanoparticles have recently been investigated extensively for their potential biomedical applications in both diagnostics and therapeutics due to their distinct physical and chemical properties compared to the bulk status. In most applications, cross-linkage of the proteins onto the nanoparticles has

utilized traditional chemical cross-linkers, which may result in unpredictable biomolecule orientation on the particle surfaces. Traditional chemical cross-linking strategies also fail to form a homogenous monolayer of ligands in most cases and thus increase the overall diameter and asymmetry of the nanoparticles. All these disadvantages lead to compromised binding and targeting efficiency.

Various metal ions have been identified to interact with a lot of different proteins [1–3]. Through a comprehensive

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Scheme 1. Simplified process for rapid adsorption and elution of 6-His tagged proteins from bacteria or cells by $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$ nanoparticles. The nanoparticles are mixed with bacteria lysate and subject to further magnetic force separation and washing to remove the remnants and the non-specific bindings. The target proteins are recovered by elution buffer containing imidazole as the competitor and the nanoparticles could be reused after re-activation. The specific affinity between 6-His tag and Ni-NTA on the nanoparticles also serves as a module for rapid orientation controlled bioconjugation for 6-His tagged functional peptides. As a demonstration, we have self-assembled RGD-4C-6-His fusion peptide on the Ni-NTA-nanoparticles which presents integrin expression specific targeting to the oral cancer cells compared to the normal oral keratinocytes.

coordination, electron-donor atoms such as S, O or N in the amino acid residues may interact with metal ions with high affinity [4, 5]. Amino acids containing electron donors include aspartate, glutamate, cysteine or histidine. Among these, histidine is a ubiquitous amino acid usually present in the metal-binding regions of metalloenzymes [6, 7]. Three-dimensional (3D) structure analysis of many metalloproteins revealed that histidine side chains usually folded into a favourable conformation of stable metal-binding pockets [8]. Although histidine may also be present on the exposed surface of many non-modified proteins, the consecutive 6–8 histidine residues automatically fold into appropriate conformation with extremely high affinity for nickel or cobalt ions compared to the single exposed histidine residue. As a result, poly-histidine residue has been one of the most widely used functional tags in molecular cloning and protein chemistry. Besides, only a few proteins in the nature have a native consecutive 6 histidine domain in their structures [9]. These advantages not only enable specific isolation of the desired peptides tagged with genetically engineered 6-histidine through a metal ion column chromatography, but also may potentially assist protein immobilization on a solid-phase support with the correct orientation for future molecule–molecule interaction based diagnostic detections or therapeutic applications [10–13].

Nitrilotriacetic acid (NTA) is a tricarboxylic acid monoamino chelator that could form a stable complex with transition metal ions such as Ni^{2+} , Zn^{2+} and Co^{2+} [14]. In the 3D structural conformation, two vacant coordination sites on the metal ion are exposed, which enable stable and strong interaction with the electron-donor containing pouch in the

poly-histidine sequences [8]. Of the metal ions, Ni^{2+} is recognized to be the best one in most applications, although recent studies have reported that the cobalt ion provides improved specificity for the 6-His tagged peptides compared to the nickel ion [15]. The correct orientation of biomolecules and the preservation of their functionality have been one of the most critical challenges for the development of nanoparticle-based biomedical applications [10, 11, 16–19]. An antibody or ligand library may thus take advantage of a self-assembly monolayer (SAM) on the particle's surface to enhance their overall performance and ease of practical usage and storage. Interestingly, there is no report on the direct synthesis of *nanoscale* aqueous $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$ particles targeting His-tagged proteins so far.

Recently, we synthesized $\text{Fe}_3\text{O}_4\text{-NH}_3^+$ nanoparticles with an average diameter of 6.2 ± 1.1 nm and demonstrated that their trypsin conjugates preserved functional activity well [20, 21]. The aqueous $\text{Fe}_3\text{O}_4\text{-NH}_3^+$ nanoparticles also displayed excellent *in vitro* biocompatibility and significantly reduced water proton relaxation times for both T_1 and T_2 . Significantly reduced T_2 and T_2^* -weighted signal intensity were observed in a 1.5 T clinical magnetic resonance (MR) imager using phantom arrays. A fast response and prolonged inverse contrast effect lasting for more than 1 week was observed in T_2^* -weighted MRI sequence of the liver of the test animal.

In this study, we provide a comprehensive and straightforward approach for the totally aqueous phase synthesis of N^α, N^α -Bis(carboxymethyl)-L-lysine (nitrilotriacetic acid; NTA) surface modified superparamagnetic Fe_3O_4 nanoparticles (scheme 1). Their efficiency and specificity in protein purification were evaluated. Hexahistidine-engineered RGD4C

peptide was synthesized to evaluate the efficiency of the selective targeting of cancer cells expressing $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin, compared to the identical nanoparticles modified by random chemical crosslinking (scheme 1). The hemolysis assay was performed using human whole blood as an initial step to validate their potential future *in vivo* applications as molecular imaging agents or targeted hyperthermia and/or chemotherapeutic agents.

2. Experimental section

2.1. Chemicals and characterization

Bromoacetic acid and N^ϵ -benzyloxycarbonyl-L-lysine were purchased from Fluka; 10% Pd/C was purchased from Lancaster; Coomassie Brilliant Blue G-250 was purchased from Bio-Rad Laboratories Inc.; PVDF membranes were purchased from PerkinElmer Inc., Boston, MA, USA; the Renaissance Western Blot Chemiluminescence Reagents were purchased from PerkinElmer Inc., Boston, MA, USA; the Scientific Imaging films were purchased from Eastman Kodak, Rochester, NY, USA; ^1H , ^{13}C NMR spectra were obtained on a 300 MHz Bruker Avance300. The thermogravimetric analysis (TGA) was obtained on a Shimadzu PA-50WSI analyser. The spectrophotometric measurements of proteins were obtained on a Dynex Technologies MRX II. The spectrophotometric measurements of hemocompatibility were obtained on a Coulter Gen-S analyser.

2.2. $\text{Fe}_3\text{O}_4\text{-NH}_3^+$ nanoparticle preparation

For preparing water-soluble and dispersed Fe_3O_4 nanoparticles, two-stage additions of protective agent and chemical coprecipitation were employed in the process [20, 21]. The 1 M ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ > 99%) and 2 M ferrous chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ > 99%) were prepared by dissolving iron salts in 2 M HCl solutions, respectively. Two solutions containing Fe^{II} and Fe^{III} at a predetermined mole ratio ($\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}} = 2$) were mixed, followed by the addition of organic acid as adherent. Subsequently, the pH of the solution was carefully adjusted to over 10 and an appropriate amount of adherent was added to achieve complete coating of the particle surface with the -NH_3^+ group, resulting in the Fe_3O_4 nanoparticle dispersion appearing as a black colour.

2.3. Synthesis of N^α, N^α -Bis(carboxymethyl)- N^ϵ -(benzyloxycarbonyl)-L-lysine (N^ϵ -Z-NTA)

Bromoacetic acid (0.52 g, 3.75 mmol) was dissolved in NaOH solution (3 ml, 5N) and cooled at 0°C . N^ϵ -benzyloxycarbonyl-L-lysine (0.42 g, 1.5 mmol) in 5 N NaOH solution (3 ml) added dropwise at 0°C while stirring [22]. After 2 h, the ice bath was removed and the mixture was refluxed overnight. The temperature of the mixture was cooled down, followed by adding a 5 N HCl solution (6 ml) with additional 0.5 h stir. The product was collected by filtration and washed by a 1 N HCl solution. N^α, N^α -Bis(carboxymethyl)- N^ϵ -(benzyloxycarbonyl)-L-lysine (N^ϵ -Z-NTA, 0.44 g) was obtained as a white solid with a yield of 78%. ^1H -NMR was performed with the following characteristics: (300 MHz,

$\text{d}_6\text{-DMSO}$) $\delta = 1.2\text{--}1.8$ (m, 6H, $\text{CHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$), $\delta = 2.9$ (q, 2H, $J = 5.9$ Hz, NHCH_2CH_2), $\delta = 3.3$ (t, 1H, $J = 7.2$ Hz, NCHCH_2), $\delta = 3.48$ (dd, 4H, $J = 21.9$, 23.3 Hz, NCH_2COOH), $\delta = 4.9$ (s, 2H, $\text{C}_6\text{H}_5\text{CH}_2$), $\delta = 7.2$ (t, 1H, $J = 5.4$ Hz, OCONH), $\delta = 7.3$ (m, 5H, $\text{C}_6\text{H}_5\text{CH}_2$); ^{13}C -NMR (75 MHz, $\text{d}_6\text{-DMSO}$) $\delta = 173.5$ (CHCOOH), $\delta = 172.8$ (CH_2COOH), $\delta = 156.1$ (OCONH), $\delta = 137.3$ (phenyl-C1), $\delta = 128.4$ (phenyl-C2, C3), $\delta = 127.8$ (phenyl-C4) $\delta = 65.1$ (NCH_2COOH), $\delta = 64.5$ (NCHCOOH), $\delta = 53.4$ (OCONHCH_2), $\delta = 29.2$ (NCHCH_2), $\delta = 29.1$ (NHCH_2CH_2), $\delta = 23.0$ ($\text{NCHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$).

2.4. Synthesis of N^α, N^α -Bis(carboxymethyl)-L-lysine(NTA)

N^α, N^α -Bis(carboxymethyl)- N^ϵ -(benzyloxycarbonyl)-L-lysine (1.09 g, 2.75 mmol) was dissolved in $\text{MeOH}/\text{H}_2\text{O}$ solution ($\text{MeOH}/\text{H}_2\text{O} = 20/1$, 40 ml) [22]. After the addition of a spatula tip of Pd/C (10%), the mixture was hydrogenated at room temperature. The reaction was completed after 3 h, as monitored by TLC ($\text{MeCN}/\text{H}_2\text{O} = 4/1$) and visualized with UV and I_2 . The catalyst was filtered off and the product was crystallized at 0°C within 2 days. The crystals (0.617 g) were collected and dried in vacuum. The final yield was calculated to be 85%. ^1H -NMR was performed with the following characteristics: (300 MHz, D_2O) $\delta = 1.3\text{--}2.0$ (m, 6 H, $\text{H}_2\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), $\delta = 2.9$ (t, 2H, NCHCH_2), $\delta = 3.8$ (s, 4H, $\text{HCNCH}_2\text{COOH}$); ^{13}C -NMR (75 MHz, D_2O) $\delta = 171.5$ (NCHCOOH), $\delta = 169.7$ (NCH_2COOH), $\delta = 67.2$ (NCH_2COOH), $\delta = 54.7$ (NCHCH_2), $\delta = 38.6$ ($\text{H}_2\text{NCH}_2\text{CH}_2$), $\delta = 26.0$ (NCHCH_2), $\delta = 25.9$ (NHCH_2CH_2), $\delta = 22.5$ ($\text{NCHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$).

2.5. Fe_3O_4 nanoparticles conjugated with NTA and Ni^{2+} ions

One millilitre of 0.1 μM (particle concentration) Fe_3O_4 nanoparticles with -NH_3 modification was added to 1 ml of 0.2 M N^α, N^α -Bis(carboxymethyl)-L-lysine (NTA) and 400 μl of 55% (w/w) glutaraldehyde at room temperature for stirring for 8 h. After conjugating Fe_3O_4 nanoparticles with NTA, 2 ml of 1.0 M NiSO_4 was added and the mixture was stirred at room temperature for different time periods. When the incubation was completed, the mixture was centrifuged at 13 000 rpm for 5 min and the supernatant was removed. The resulting precipitates ($\text{Fe}_3\text{O}_4\text{-NTA-Ni}$) were redispersed in 2 ml H_2O and centrifuged at 13 000 rpm for 5 min to remove the excess Ni^{2+} ions. The $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$ nanoparticles were further redispersed in 1 ml H_2O and analysed by atomic absorption spectroscopy (AA) to measure the ratio of Ni^{2+} ion to $\text{Fe}_3\text{O}_4\text{-NTA}$ nanoparticles as a function of incubation period between NiSO_4 and $\text{NTA-Fe}_3\text{O}_4$.

2.6. The quantity of NTA molecules on Fe_3O_4 nanoparticles

The quantity of NTA molecules on the Fe_3O_4 nanoparticle's surface was measured and evaluated by thermogravimetric analysis (TGA). The experiments were carried out using 14.79 mg of $\text{Fe}_3\text{O}_4\text{-NTA}$ sample, which was placed in an open Pt pan. Compressed air flowing at a rate of 20 ml min^{-1} served as the purge gas and the sample temperature was raised from 26.8 to 600°C at a rate of $10^\circ\text{C min}^{-1}$. Based on the loss of sample weight, we can calculate the quantity of NTA molecules conjugated with the Fe_3O_4 nanoparticles.

2.7. Determination of the binding capacity for 6-His tagged proteins

The binding capacity of the newly synthesized $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$ nanoparticles to 6-His tagged proteins was evaluated by using purified V344A mutant streptopain (M_w 42 kDa) with 6-His tag on the N-terminus [23]. For the protein binding assay, the purified streptopain solutions ($350.86 \mu\text{g ml}^{-1}$) varied from 0 to $390 \mu\text{l}$ were mixed with $10 \mu\text{l}$ of $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$ nanoparticles (32 mg ml^{-1}), then added to the binding buffer to a total volume of $400 \mu\text{l}$. Subsequently, the reaction mixtures were incubated at 4°C for 20 min. The nanoparticles with adsorbed streptopain were isolated by a small magnet. The nanoparticles were washed with $100 \mu\text{l}$ wash buffer ($200 \text{ mM NaH}_2\text{PO}_4$, 500 mM NaCl , pH 7.4) to eliminate non-specific bindings. The flow-through solution and washing solutions were collected to detect the non-binding proteins and to calculate the quantity of the binding proteins on $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$. For the quantification of the purified streptopain, Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories Inc.) was applied as the dye to visualize the proteins. The binding capacity was determined by spectrophotometric measurement of the optical adsorption (590 nm) of each fraction of the collected solutions and converted into protein concentrations according to the standard curve using BSA as the reference protein.

The effect of elution buffers to the final yield of protein purification was evaluated as a function of imidazole concentration. The protein binding and wash procedure were performed as the aforementioned text except for the ingredients of the final elution buffers, which contained different concentrations of imidazole. The final yield of the target protein was evaluated by spectrophotometric analysis at 590 nm.

2.8. Purification of 6-His tagged recombinant proteins from bacteria lysate

The *E. coli* BL21 (DE3) transfected with pET21a containing a full-length mutant streptopain cDNA V334A with 6-his tagged on the N-terminal end [23]. Cells were grown at 37°C for 6–8 h in LB medium (1 l of 10 g of Bacto-Trypton, 5 g of Bacto-yeast extract, and 10 g of NaCl adjusted to pH 7.2 with 3N NaOH). A final bacteria concentration at $A_{600} = 0.5\text{--}1.0$ was used to induce recombinant gene expression by the addition of isopropyl-1-thio- β -D-galactopyranoside (IPTG) to a final concentration of 1 mM and further incubated for additional 24 h at 37°C . The bacteria was collected by centrifugation and lysed by liquid shear with a French press to obtain the final lysate on ice, and then the lysate was centrifuged ($13\,000 \text{ rpm}$, for 10 min at 4°C) to remove the crude precipitates before being subject to further protein purification.

To evaluate the efficiency of protein purification utilizing newly synthesized NTA-Ni modified magnetite nanoparticles through magnetic force manipulation, the supernatant of cell lysate was mixed with a buffer solution containing the $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$ nanoparticles at a concentration of 32 mg ml^{-1} followed by a magnetic separation. The protein purification was performed as described earlier. The derived proteins in each fraction were visualized on a SDS-PAGE after electrophoresis and Coomassie Brilliant Blue G-250 staining.

2.9. Western blot analysis

To evaluate the specificity and degradation in the elution process of the target protein, Western blot analysis was performed. $20 \mu\text{l}$ of each collected fraction was loaded on a 15% SDS-polyacrylamide gel and separated by electrophoresis, then transferred to a PVDF membrane (PerkinElmer Inc., Boston, MA, USA). The streptopain was detected with a horse radish peroxidase conjugated monoclonal antibody against 6-His-tag of the fusion protein (Chemicon International Inc., Temecular, CA, USA). After incubation, the signal was detected by chemiluminescence assay using the Renaissance Western Blot Chemiluminescence Reagent (PerkinElmer, Inc., Boston, MA, USA) and Scientific Imaging film (Eastman Kodak, Rochester, NY, USA).

2.10. Hemocompatibility of $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$ nanoparticles

Spectrophotometric measurement for the presence of hemoglobin in human plasma released from ruptured red blood cells after exposure to the nanoparticles served as an index for their hemocompatibility. The fresh human whole blood was obtained from a healthy donor with informed consent, following guidelines for studies using human tissues. $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$ nanoparticles were added to 1 ml of human whole blood containing 12 IU sodium heparin to final particle concentrations of 0, 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , and 10^{-5} M . The solutions were gently mixed on a rotary shaker for 4 h at 37°C followed by centrifugation at 1200 rpm for 10 min to remove the red blood cells and another centrifugation at 13000 rpm for 30 min to eliminate the nanoparticles. The hemoglobin concentration in the supernatant was then determined by spectrophotometric analysis (454/528 nm) in a Coulter Gen-S analyser (Beckman Inc, USA).

As a comparative study, we also evaluated the relative hemolysis of the nanoparticles at different concentrations compared to the positive and the negative control. Fresh human blood was collected and diluted with normal saline solution (8 ml blood + 10 ml normal saline). The hemolysis assay was performed by adding $20 \mu\text{l}$ of the diluted blood to 1 ml of 0.1% sodium carbonate solution as a positive control or 1 ml of normal saline as a negative control or 1 ml of the experimental groups containing different concentrations of the nanoparticles, ranging from $10^{-6}\text{--}10^{-10} \text{ M}$. After incubation, all the test tubes were centrifuged for 5 min at 3000 rpm and the supernatant was transferred to a cuvette for spectroscopic analysis at a wavelength of 545 nm, and the percentage of hemolysis was calculated by:

Percentage hemolysis

$$= \frac{[\text{OD}(\text{test}) - \text{OD}(\text{negative control}) \times 100]}{[\text{OD}(\text{positive control}) - \text{OD}(\text{negative control})]}$$

where OD = the optical density at 545 nm.

The percentage of hemolysis was calculated based on the average of three replicates. High hemocompatibility was given for samples with <5% hemolysis, hemocompatible for samples within 10% hemolysis, and non-hemocompatible for >20% hemolysis.

Scheme 2. The surface modification of the Fe_3O_4 -NTA-Ni nanoparticle complex. A covalent bond is established between the primary amine of the nanoparticle surface and glutaraldehyde. The NTA covalently attaches onto the nanoparticle with a projected carboxyl group to chelate the nickel ion and leaves two vacant sites for affinity binding to 6-His tagged proteins.

2.11. Preparation of RGD-4C-6-His magnetite nanoparticle conjugate and *in vitro* targeting analysis

The RGD-4C-6-His peptide (H-Ala-Cys-Asp-Cys-Arg-Gly-Asp-Cys-Phe-Cys-Gly-Gly-Gly-Gly-Gly-His-His-His-His-His-His-OH, disulfide bridge: 2–10 and 4–8) was synthesized (Genesis Biotech Inc., Taiwan) for the evaluation of cell targeting. The resulting Fe_3O_4 -NTA-Ni reserves two binding sites for the interaction with the poly-histidine structure of the fusion peptide (scheme 2). Two vacant coordination sites on the nickel ions enable strong complexing with the electron-donor containing pouch in the poly-histidine sequences. For conjugation of the RGD-4C-6-His peptide to the nanoparticle surface, the peptide was incubated with the particle at room temperature for 5 min under gentle agitation in a 10:1 peptide-to-nanoparticle molar ratio, then subjected to magnetic separation and washing to remove non-binding peptides. The particles were re-suspended in phosphate buffer containing 10% goat serum and sonication in a water bath for 10 min before the experimental targeting. Oral cancer cell line HCDB-1 [24], which expresses a high level of $\alpha v \beta 3$ integrin and normal oral keratinocytes (HNOK) with lower expression level, served as the model system and the negative control for a selective targeting experiment, respectively. The cells were grown in chamber slides (Nalge Nunc International Corp., IL, USA) in their respective culture media at 37°C in humidified air containing 5% CO_2 . The RGD-4C-6-His magnetite nanoparticles were then co-incubated with cells cultured on the chamber slides for 30 min at 4°C with gentle shaking. The medium was removed and washed twice with PBS, followed by fixing with 4% glutaraldehyde. To visualize the nanoparticle aggregates caused by affinity binding between RGD-4C and integrin on the target cells, Pearl's iron staining was performed and the results were photographed under an optical microscopy (Olympus, BH-2).

2.12. Instrumentation

Electron micrographs of the Fe_3O_4 dispersions were carried out using a drop of the sample onto a copper mesh coated with an amorphous carbon film, and then dried in a vacuum desiccator. The prepared samples were studied in a transmission electron microscope (JEOL JEM-1200-EX). Thermogravimetric analysis was carried out under a stream of nitrogen, at a heating rate of $10^\circ\text{C min}^{-1}$, using a TA Q50 Thermogravimetric analyser (TA Instruments Inc.).

Figure 1. TEM image of Fe_3O_4 nanoparticles conjugated with NTA.

3. Results and discussion

3.1. Preparation of $\text{Fe}_3\text{O}_4\text{-NH}_3^+$ modified with NTA and Ni^{2+} ion

We have recently reported the aqueous synthesis of $\text{Fe}_3\text{O}_4\text{-NH}_3^+$ nanoparticles (6.2 ± 1.1 nm) and applied enzyme bio-conjugation, such as trypsin [20, 21]. This strategy for enzyme immobilization could preserve the enzyme activity. Furthermore, we demonstrated the negative contrast effect for MR imaging applications and their biocompatibility [21]. Herein, the Fe_3O_4 nanoparticle surface with amine functional groups is further covalently crosslinked to NTA by glutaraldehyde (scheme 2).

Scheme 2 illustrates the surface chemistry of the newly synthesized nanoparticles. The NTA was covalently attached to the amino groups on the nanoparticles by using glutaraldehyde as the linker. Figure 1 displays the TEM image of as-prepared Fe_3O_4 -NTA nanoparticles with some degree of aggregation. The nickel ions were chelated by NTA molecules immobilized on the nanoparticle surface. The resulting Fe_3O_4 -NTA-Ni reserves two binding sites for the interaction with the poly-histidine structure of the fusion peptide (scheme 2), where two vacant coordination sites on the nickel ions are exposed and enable strong coordination with the electron-donor containing pouch in the poly-histidine sequences.

Figure 2. The thermogravimetric analysis (TGA) curve for $\text{Fe}_3\text{O}_4\text{-NTA}$ (inset: TGA behaviour from pure NTA molecules).

TGA analysis was carried out and the quantity of the NTA conjugation was evaluated from the weight lost in the TGA curve. The TGA profile shows that the weight loss occurs in steps, with total loss of 26% up to 600 °C (figure 2). The initial weight loss of ~6.5% can be attributed to the elimination of physical and chemical adsorbed H_2O between 30 and 160 °C. The weight loss of ~4.7% in the temperature range 160–210 °C is assigned as the decomposition of the linker, glutaraldehyde. The glutaraldehyde molecules have been found to decompose at a temperature of ~188 °C [25]. A pronounced weight loss step is found in the temperature range from 210 °C up to 600 °C, which can be ascribed to the decomposition of the NTA molecules and is consistent with the TGA behavior of the pure NTA molecules (inset in figure 2). It is evaluated that a weight loss of ~14.8% is determined and approximately 340 NTA molecules are tagged on a Fe_3O_4 nanoparticle.

The adsorption of Ni^{2+} onto $\text{Fe}_3\text{O}_4\text{-NTA}$ was monitored as a function of incubation time. The ratio of Ni^{2+} and Fe_3O_4 was calculated based on the analysis of atomic absorption. Adsorption studies of nickel ions indicate that the ratio of Ni^{2+} binding on the $\text{Fe}_3\text{O}_4\text{-NTA}$ nanoparticles rapidly increased from 0 to 80 over the initial incubation time of 2 h. Then the adsorption ratio gradually rose to ~140 when the incubation was extended for 8 h (figure 3). After incubation for 10 h, the intensity increased only slightly to ~154, which suggests that the binding capacity of $\text{Fe}_3\text{O}_4\text{-NTA}$ should have reached saturation for the nickel ions.

3.2. Purification of 6-His tagged recombinant proteins from bacteria lysate

The binding capacity of the $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$ to 6-His tagged proteins was evaluated by using purified recombinant 6-His

Figure 3. Adsorption of nickel ions onto the $\text{Fe}_3\text{O}_4\text{-NTA}$ nanoparticles as a function of incubation time between NiSO_4 and $\text{Fe}_3\text{O}_4\text{-NTA}$.

Figure 4. The ratio of protein to $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$ nanoparticles as a function of protein dosage. The total amount of the nanoparticles remained constant throughout the experiments ($32 \text{ mg}^{-1} \text{ ml}^{-1}$ for $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$).

tagged V344A mutant streptopain (42 kDa). Figure 4 shows that the protein binding efficiency gradually increased from the initial 0.01 to 0.23 (mg protein per mg $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$). In these results, we found that the binding capacity of $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$ nanoparticles is about 45 times higher than that of the commercial magnetic microbeads (Dynabead®, 5–12 µg protein per mg bead), which indicates that the high surface/volume ratio of the nanoparticles may contribute to the higher efficiency. The optimal performance of the protein release was investigated by modulation of imidazole concentration in elution buffer. As shown in figure 5, the eluted efficiency reaches a plateau as the imidazole concentration approaches 300 mM. When the imidazole concentration in the elution buffer is higher than 500 mM, the excess imidazole would interfere with the spectrophotometric analysis of the protein. Thereafter, a 400 mM of imidazole concentration is chosen for the following protein purification.

As the synthesized $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$ nanoparticles are able to bind and release purified 6-His-tagged peptides, we further evaluate the direct protein purification from bacteria expressing mutant streptopain with 6-His tagged on the N-terminus. The process is illustrated in scheme 1. Figure 6(a) shows the SDS-PAGE analysis of the products from each fraction of the

Figure 5. The 6-His protein desorption as a function of imidazole concentration.

Figure 6. (a) SDS-PAGE analysis reveals a specific binding of the Fe_3O_4 -NTA-Ni nanoparticles to the streptopain mutant protein with 6-His tag from a crude bacteria lysate following magnetic force separation. (b) Western blot analysis probed by anti-6-His monoclonal antibody shows a specific high yield of the protein following the magnetic purification protocol.

elution process. A prominent band at a molecular weight of 42 KDa (the molecular weight of streptopain) was observed in the original bacteria total lysate (lane 1), and the first (lane 4) and second elution (lane 5), but was not detectable in the pass through (lane 2) and wash buffer (lane 3) collections. In

addition, Western blot analysis also demonstrated a single band with the correct molecular weight of the protein. Significant protein recovery without evidence of degradation and non-specific binding was observed (figure 6(b)). Furthermore, thermal stability of the Fe_3O_4 -NTA-Ni nanoparticles was observed, as they can be stored at a temperature of 50 °C for more than one month and still retain their functionality.

Since the presented iron oxide particles are fabricated in an aqueous environment, a similar idea for modulated design of SAM on nanoparticles such as conjugation of glutathione for the purification of GST-fusion proteins could be achieved and is currently under investigation.

3.3. The RGD-4C-6-His magnetite nanoparticle conjugate and *in vitro* targeting analysis

The specific affinity between 6-His-tagged peptides and NTA-Ni establishes a platform to engineer precisely the oriented biomolecules on nanoparticle surfaces, where the conventional chemical crosslinking strategies usually result in a randomized orientation in biomolecules. This enables enhanced *in vivo* targeting for the molecular diagnosis or therapeutic applications.

For further *in vitro* or *in vivo* studies, a primary evaluation of the hemocompatibility was conducted by hemolysis assay for Fe_3O_4 -NTA-Ni. The hemolysis assay shows no detectable hemolysis activity of the nanoparticles at all dosages ranging from 0.1 mM to 10 mM particle concentrations, as seen in figure 7(a). In addition, the potential hemolysis behaviour was compared to the positive (0.1% sodium carbonate) and the negative control (normal saline) after incubation for 1, 2 and 4 h. As shown in figure 7(b), all test dosages present no significant hemolysis (<1% of the control).

Cancer cells and their neoangiogenic blood vessels usually present altered expression of cell adhesion molecules including specific types of integrins [26]. Cyclic RGD-4C peptide presents specific affinity to $\alpha_v\beta_3$ and $\alpha_3\beta_5$ integrins that often selectively express in tumor tissues. Enhanced tumor gene therapy has been demonstrated using RGD-4C modified adenovirus [27]. This peptide has also been applied for the imaging and monitoring of tumor angiogenesis [28, 29]. Herein, we design a fusion peptide consisting of a cyclic

Figure 7. (a) Hemocompatibility assay of the as-synthesized Fe_3O_4 -NTA-Ni nanoparticles performed in freshly prepared human whole blood by measuring the presence of hemoglobin in the serum derived from lysed red blood cells upon exposure to the nanoparticles. No significant hemolysis activity is detected in all dosages compared to the control set of the phosphate buffer saline control (0.0 M particle concentration). (b) The Fe_3O_4 -NTA-Ni nanoparticles show no significant hemolysis detected during time periods of 1, 2 and 4 h of exposure in all test dosages, with reference to the positive control (0.1% sodium carbonate).

Figure 8. The RGD4C-6His cyclic peptides self-assembled on $\text{Fe}_3\text{O}_4\text{-NTA-Ni}$ nanoparticles present no detectable binding to the normal oral keratinocytes (a) compared to the oral cancer cell HCDB1 (b), as revealed by iron staining (blue colour). Fe_3O_4 nanoparticles with traditional chemical crosslinking of the RGD4C-6His cyclic peptides (c) show significantly less efficient targeting than (b). Nanoparticles alone fail to target HCDB1 cells and present only background staining (d). (Scale bar = 20 μm .)

RGD-4C domain, a 6 glycine spacer and a 6-His tag on the C-terminal end. Oral cancer cells (HCDB-1) [24] expressing a high level of $\alpha_v\beta_3$ integrin compared to the normal oral keratinocytes (HNOK) are used for the *in vitro* targeting. Nanoparticles were incubated with the peptides before incubation with either fixed or living cells cultured on a chamber slide. The results show a prominent selectively targeting of the nanoparticle-RGD-4C-6-His complex to the cancer cells (figure 8(b)), while only little non-specific staining was detectable in the normal keratinocytes (figure 8(a)). In a control experiment without molecular orientation, we synthesized RGD-4C surface modified nanoparticles using a simply applied chemical crosslinking method using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) to form a covalent bond between the amine group on the nanoparticle surface and the carboxyl group on the peptide. As shown in figure 8(c), the randomly crosslinking RGD-4C without molecular orientation revealed significantly less efficient targeting than the self-assembled RGD-4C-nanoparticles in figure 8(b). Finally, figure 8(d) shows that nanoparticles alone failed to target HCDB1 cells and showed only background staining. These results suggest the advantage of the self-assembly strategy through the ‘key’ (NTA-Ni) to ‘key lock’ (6-His tag on the targeting biomolecule warhead) concept, which provides enhanced targeting efficiency due to the precisely controlled biomolecular orientation on the nanoparticle.

4. Conclusions

We have demonstrated the aqueous synthesis of $\text{Fe}_3\text{O}_4\text{-NH}_3^+$ nanoparticles modified with NTA-Ni. The modified nanoparticles show high efficiency and specificity for the rapid purification of 6-His tagged proteins and present no hemolysis between concentrations of 10^{-5} – 10^{-10} M as well as satisfactory biocompatibility for *in vitro* studies. *In vivo* targeting assay showed significantly enhanced tumor cell targeting compared to random chemical conjugation. These results indicate that the as-synthesized iron oxide nanoparticles may hold great potential as universal modularly designed functionalized carriers for magnetite field guided biomolecule purification, magnetic force driven drug delivery nanovehicles, and molecular imaging agents, which present the advantages of modularly designed particle and targeting moiety with

guaranteed biomolecule orientation. The platform could be applied further to a broad spectrum of affinity-based protein–protein interaction biosensing.

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References

- [1] Zaric S D, Popovic D M and Knapp E W 2000 *Chem. Eur. J.* **6** 3935
- [2] Rosenzweig A C, Huffman D L, Hou M Y, Wernimont A K, Pufahl R A and O’Halloran T V 1999 *Struct. Fold Des.* **7** 605
- [3] Ha Y, Shi D S, Small G W, Theil E C and Allewell N M 1999 *J. Biol. Inorg. Chem.* **4** 243
- [4] Hutchens T W and Yip T T 1991 *J. Chromatogr.* **536** 1
- [5] Kumar A, Galaev I Y and Mattiasson B 1998 *Biotechnol. Bioeng.* **59** 695
- [6] Chen H T, Legault P, Glushka J, Omichinski J G and Scott R A 2000 *Protein Sci.* **9** 1743
- [7] Teng H and Grubmeyer C 1999 *Biochemistry* **38** 7363
- [8] Bhattacharyya R, Saha R P, Samanta U and Chakrabarti P 2003 *J. Proteome. Res.* **2** 255
- [9] Delaney S J, Hayward D C, Barleben F, Fischbach K F and Miklos G L 1991 *Proc. Natl Acad. Sci. USA* **88** 7214
- [10] Cox J H, Ivanyi J, Young D B, Lamb J R, Syred A D and Francis M J 1998 *Eur. J. Immunol.* **18** 2015
- [11] Joyce S, Sun R and Nathenson S G 1992 *Biochem. Biophys. Res. Commun.* **186** 1449
- [12] Peterson E M, Cheng X, Qu Z H and DelaMaza L M 1996 *Mol. Immunol.* **33** 335
- [13] Fochoer F, Spadari S and Maga G 2003 *Curr. Drug. Targets Infect. Disord.* **3** 41
- [14] Juang R S and Wang Y C 2003 *Water Res.* **37** 845
- [15] Phan T C, Nowak K J, Akkari P A, Zheng M H and Xu J 2003 *Protein Expr. Purif.* **29** 284
- [16] Hainfeld J F, Liu W Q, Halsey C M R, Freimuth P and Powell R D 1999 *J. Struct. Biol.* **127** 185
- [17] Buchel C, Morris E, Orlova E and Barber J 2001 *J. Mol. Biol.* **312** 371
- [18] Abad J M, Mertens S F L, Pita M, Fernandez V M and Schiffrin D J 2005 *J. Am. Chem. Soc.* **127** 5689

- [19] Gu H, Ho P L, Tsang K W T, Wang L and Xu B 2003 *J. Am. Chem. Soc.* **125** 15702
- [20] Yeh C S, Cheng F Y, Shieh D B and Wu C L 2004 *ROC Patent Specification* 202070, *German Patent* submitted
- [21] Shieh D B, Cheng F Y, Su C-H, Yeh C S, Wu M T, Wu Y N, Tsai C Y, Wu C L, Chen D H and Chou C H 2005 *Biomaterials* **26** 7183
- [22] Lutz S, Christian D and Tampe R 1994 *J. Am. Chem. Soc.* **116** 8485
- [23] Chen C Y, Luo S C, Kuo C F, Lin Y S, Wu J J, Lin M T, Liu C C, Jeng W Y and Chuang W J 2003 *J. Biol. Chem.* **278** 17336
- [24] Lin S C, Chang K W, Chang C S, Yu S Y, Chao S Y and Wong Y K 2000 *Proc. Natl Sci. Counc. Rep. China B* **24** 129
- [25] Lide D R 2000 *CRC Handbook of Chemistry and Physics* 81th edn (London: CRC Press)
- [26] Zitzmann S, Ehemann V and Schwab M 2002 *Cancer Res.* **62** 5139
- [27] Bauerschmitz G J *et al* 2002 *Cancer Res.* **62** 1266
- [28] Su Z F, Liu G, Gupta S, Zhu Z, Rusckowski M and Hnatowich D J 2002 *Bioconjug. Chem.* **13** 561
- [29] Chen X, Sievers E, Hou Y, Park R, Tohme M, Bart R, Bremner R, Bading J R and Conti P S 2005 *Neoplasia* **7** 271