ACS APPLIED MATERIALS & INTERFACES

Letter

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High Throughput Detection of Human Neutrophil Peptides from Serum, Saliva, and Tear by Anthrax Lethal Factor-Modified Nanoparticles

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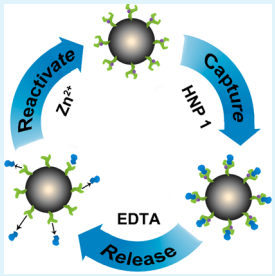
Supporting Information

ABSTRACT: Human α defensins human neutrophil peptide 1−3 (HNP 1−3) are potential prognostic cancer biomarkers. Metalloprotein anthrax lethal factor (ALF) binds to HNP 1−3 in a Zn^{2+}-dependent manner. We conjugated ALF to the surface of magnetic nanoparticles (MNP) to magnetically isolate the HNPs, and used Zn^{2+} to control the capture and release HNPs.



KEYWORDS: *defensin, anthrax lethal factor, magnetic nanoparticles, MALDI-TOF-MS*

1.. INTRODUCTION

The occurrence and progression of tumor are closely related to the development of tumor microenvironment,[1] thus studies focusing on tumor microenvironment have attracted widespread attentions.[2] The expressions of proteins and peptides in tumor microenvironment are usually cancer relevant,[3] and many of these molecules are being investigated as potential cancer-related biomarkers, derived either from tumor cells or normal cells in the tumor microenvironment. Biomarkers derived directly from tumor cells usually mutate with the progression of tumor, while biomarkers originated from the inflammatory cells in the tumor microenvironment are from normal cells, which do not mutate with the development of the disease, and are closely associated with specified type of cancer. Among these biomarkers, we are particularly interested in the potential prognostic biomarkers human defensins HNP 1−3.[4−6] Compared to other complicated cancer-type specific biomarkers, HNP 1−3 have been explicitly proposed as signature biomarkers universal to many cancers.[7−11] The abundance assessments of HNP 1−3 can help to delineate pathogenesis at molecular level and will provide more valid information for clinical diagnosis. However, the implementation of HNP 1−3 as biomarker has been hampered by the current tedious separation techniques, such as HPLC, which is not a high-throughput assay and requires complicated sample pretreatment,[12] and ELISA assay, which requires multiple steps and is sometimes not approachable because of the availability or stability of the antibodies.[13,14] In this study, we seek to develop a method for robust, convenient, and high-throughput separation of HNP 1−3.

Anthrax lethal factor (ALF) is a relatively stable metalloprotein, whose structure and activity is Zn^{2+}-dependent.[15,16] HNP 1 can specifically bind to a region remote from the active site of the ALF protein, with high affinity,[17] and the association is not interfered by the formation of lethal toxic complex (LeTx) between ALF and protective antigen (PA), regardless of the complicated physiological environments in vitro or in vivo.[18] It is intriguing that the binding and release of HNP 1−3 from ALF is reversible and Zn^{2+} dependent, i.e., removal of Zn^{2+} by chelating agent such as EDTA can readily inactivate ALF and release the previously bound HNP 1−3 molecules; adding Zn^{2+} can reactivate the ALF and restore its ability to bind free HNP 1−3 molecules. This reversible association between ALF and HNP 1−3 can be implemented to achieve repeatable separation of HNP 1−3.

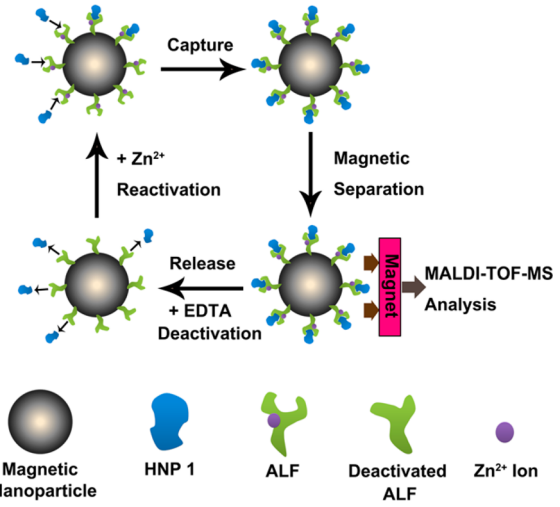
Rapid and specific separation of HNPs from body fluid by ALF modified matrix is key for our design of using ALF to enrich HNP 1−3. Magnetic separation is quite unique and convenient among separation techniques, and has been applied in different fields of life science study such as microbiology, immunology and molecular biology.[19] Robust, highly convenient and easily automatable protocols make it ideal for isolating HNP 1−3 from fluid when ALF is immobilized onto superparamagnetic nanoparticle (MNP), such as Fe_3O_4 nanoparticles, which is easy to prepare and make surface modification.[20−23]

Received: June 4, 2013
Accepted: August 21, 2013
Published: August 21, 2013

dx.doi.org/10.1021/am4021523 | ACS Appl. Mater. Interfaces 2013, 5, 8267−8272

In this study, ALF-modified MNP (Fe_3O_4@ALF, AMNP) was readily prepared and used for the enrichment of HNP 1 from buffer and body fluids (tear, saliva and serum). As demonstrated in Scheme 1, AMNPs were dispersed into fluid

Scheme 1. Schema of Anthrax Lethal Factor (ALF) Modified Magnetic Nanoparticles (MNPs) Used for Repeat Enrichment of HNP 1 from Sample



to enrich HNP 1; the resulting ALF-HNP 1 complex can be rapidly separated from solution by applying magnetic field; HNP 1 can be released upon removal of Zn^{2+} by EDTA, and further analyzed by HPLC for accurate quantification; the ALF modified AMNPs can be reactivated by adding Zn^{2+} and reused to collect HNPs for the next round. Compared with conventional separation techniques like HPLC, the separation and detection of HNP was greatly simplified by AMNPs.

2.. EXPERIMENTAL SECTION

Materials and Instruments. $FeCl_2{\cdot}4H_2O$, $FeCl_3{\cdot}6H_2O$, octanedioic acid were purchased from Bomaijie Inc. (Beijing, China). Ammonium hydroxide (28−30 wt %), tetraethylothosilicate (TEOS), (3-aminopropyl) triethoxysilane (APS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anthrax lethal factor (Cata. No. 176900−100UG) was purchased from Merck KGaA (Darmstadt, Germany). Water was purified from a Milli-Q system (Millipore, Milford, MA, USA). Human serum (HS),[24] saliva[25] and tear[26] were collected from healthy volunteers according to IRB approved protocols. Briefly, salivary samples were collected through unstimulated method. Volunteers were instructed to rinse the mouth thoroughly with Milli-Q water before salivary sample collection. Then, whole saliva was allowed to drip off the lower lip into the prepared Eppendorf tubes. Human serum was obtained by collecting blood from volunteers by red top blood collection tubes. After the clot formation, tubes were centrifuged for 20 min at 1100−1300 g and the serum was transferred into labeled cryovials. The tear samples were collected using sterilized pipet to gently collect the samples from each eye separately from the inferior conjunctival fornix and/or lacus lacrimalis into prepared Eppendorf tubes.

MALDI-TOF MS (Microflex LRF, Bruker Daltonics), Physical performance analyzer (PPMS-9, Quantum Design Inc.), FTIR spectroscopy (Spectrum one, Perkin-Elmer), Zetasizer (Nano ZS90, Malvern), transmission electron microscope (TEM, Tecnai G2 20 S-TWIN).

Preparation of Fe_3O_4 Nanoparticles. 540 mg (3.33 mmol) $FeCl_3$, 210 mg (0.89 mmol) $FeCl_2{\cdot}6H_2O$ were dissolved in 52 mL Milli-Q water and mixed at 700 rpm completely. Then 48 mL 2.9% $NH_3{\cdot}H_2O$ (0.83 M) was added into the mixture under the protection of dry N_2. The reaction was stopped after 30 min and the product was ripened in 60 °C water bath for additional 30 min. After that, the resulted Fe_3O_4 nanoparticles were collected by magnet, washed 6 times with Milli-Q water, resuspended in water, and then the volume was set to 100 mL.

Preparation of Fe_3O_4@SiO_2 Nanoparticles. Fifty milliliters of Milli-Q water was added into 50 mL of aqueous Fe_3O_4 nanoparticle suspension, and then 20% TEOS acetic acid solution was added and the reaction was maintained for 3 h. After that, 80 μL (0.382 mmol) of TEOS and 20 μL of ethanol were added into the solution and reacted for additional 24 h under N_2 protection. The product was collected by magnet and washed 6 times with Milli-Q water. The resulted Fe_3O_4@SiO_2 nanoparticles were suspended in Milli-Q water (volume was set to 50 mL) and kept for further synthesis.

Preparation of Fe_3O_4@APS Nanoparticles. 75 mL Milli-Q water was added into 25 mL of aqueous Fe_3O_4@SiO_2 nanoparticle solution and completely mixed. Then, 100 μL (0.573 mmol) of APS was added and reacted for 48 h under N_2 protection. The product was collected by magnet and washed 6 times with Milli-Q water. The resulted Fe_3O_4@APS nanoparticles were suspended in 5 mL of water and stored for further synthesis.

Preparation of Fe_3O_4@COOH Nanoparticles. One-hundred twenty-one milligrams (0.7 mmol) of suberic acid and 26.84 mg (0.14 mmol) of EDC were dissolved into PBS buffer (pH 7.4), then 0.5 mL Fe_3O_4@APS nanoparticle aqueous solution was added. After 15 min, 32 mg (0.28 mmol) of NHS was added into the mixture and the reaction was kept for 24 h. The product Fe_3O_4@COOH nanoparticles were collected by magnate and washed 6 times with Milli-Q water, then resuspended in 5 mL of water.

Preparation of Fe_3O_4@ALF Nanoparticles (AMNPs). Fifty-four milligrams of (0.28 μmol) EDC, 15.6 μg (0.17 nmol) ALF, 32 μg (0.28 μmol) of NHS, 46 μL (0.46 μmol) of β-aminoethoxyethanol, and 20 μL of aqueous Fe_3O_4@COOH nanoparticle solutions were mixed thoroughly and stirred for 24 h. Then the resulting Fe_3O_4@ALF nanoparticles (AMNPs) were collected by magnet and washed 6 times with Milli-Q water. The product was suspended in 20 μL water for further experiments.

Sample Analysis. One μL AMNPs were added into sample and mixed thoroughly. After fully reacting with HNP 1, nanoparticles were collected by magnet and washed 9 times with Milli-Q water. The supernatants and nanoparticles were subjected for MALDI-TOF-MS analysis. Sample solutions like aqueous solution, FBS solution, and HS solution were added with known amount of HNP 1, whereas saliva and tear samples were analyzed as they were originally collected.

Recycled Analysis of HNP 1 Samples. Release HNP 1 from AMNP: 2 μL EDTA (1 mg/mL) was added into AMNPs with captured HNP 1, and then the nanoparticles were collected by magnet after the conjugated ALF being deactivated. The EDTA treatment was processed for 5 times

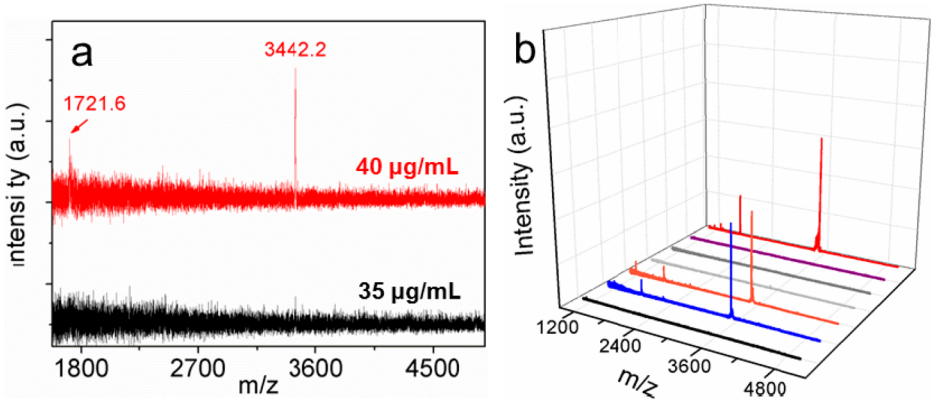


Figure 1. MALDI-TOF-MS spectra: (a) HNP 1 with concentration of 40 μg/mL and 35 μg/mL; (b) supernatant of HNP 1 solution after AMNP treatment (black), HNP 1 collected by AMNP (blue), released HNP 1 from EDTA deactivated AMNPs (rose), almost no residual HNP 1 on EDTA-deactivated AMNP (light gray), reactivated AMNP with 1 mM Zn^{2+} (medium gray), the supernatant of HNP 1 solution after being treated by the regenerated AMNP (purple), and the adsorbed HNP 1 by the reactivated AMNPs (red). For all these assays, the sample volume used in MALDI-TOF is 1 μL.

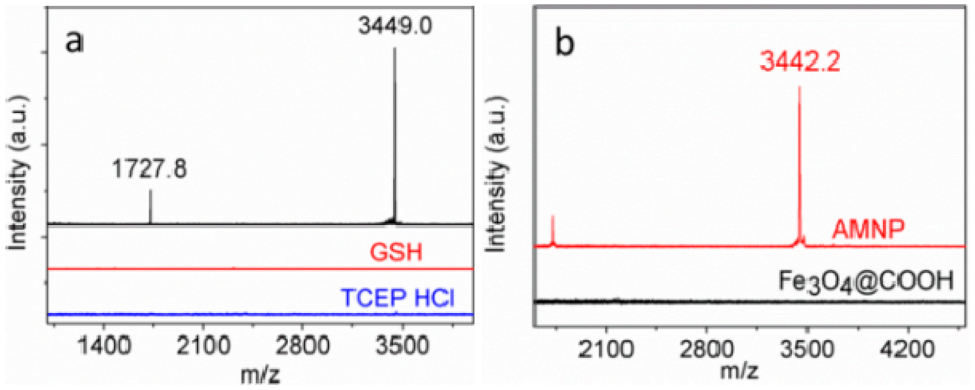


Figure 2. MALDI-TOF-MS spectra: (a) reduced HNP 1 in TCEP solution (black), AMNPs collected from solution of reduced HNP 1 with TCEP (blue), and AMNPs collected from solution of reduced HNP 1 with GSH (red); (b) AMNPs collected from HNP 1 solution (red), and Fe_3O_4@COOH nanoparticles collected from HNP 1 solution.

and all the supernatants were collected for MALDI-TOF-MS analysis.

Reactivation of ALF. Two microliters of Zn^{2+} solutions (1 mM) were mixed with the previous EDTA treated AMNPs, and the nanoparticles were collected by magnate. The Zn^{2+} treatment was repeated 3 times and all the supernatants were collected for MALDI-TOF analysis.

Sample Analysis. The reactivated AMNPs with associated HNP 1 were then subjected for HNP 1 sample analysis. The whole recycled analysis process was repeated for 3 times.

3.. RESULTS AND DISCUSSION

Fe_3O_4 nanoparticles with size around 10 nm were fabricated by coprecipitation method (see Figure S1a in the Supporting Information). Magnetic Fe_3O_4@SiO_2 particles with multidoped Fe_3O_4 cores were prepared from traditional sol−gel method (see Figure S1b in the Supporting Information). The silica surface was modified by APS, and sequentially modified with suberic acid and ALF. The ζ potential changed with the introduction of different surface chemistry. The ζ potential values of nanoparticles, i.e., Fe−OH (−7.8 mV), Si−OH (−35 mV), $-NH_2$ (16 mV), and −COOH (−10 mV), were obtained at neutral pH, respectively (see Figure S2a in the Supporting Information). The corresponding signature IR absorptions also appeared along the process of modification (1047 cm^{-1}, ν Si−O−Si of Fe_3O_4@SiO_2; 1511 cm^{-1}, δ NH, 1468 cm^{-1}, ν CH_2 of Fe_3O_4@APS; 1528 cm^{-1}, amide II band; and 1406 cm^{-1}, ν C−O of Fe_3O_4@COOH) (see Figure S2b in the Supporting Information).[27] Besides, the superparamagnetic character of Fe_3O_4 nanoparticles was retained along with each sequential doping step, from SiO_2 coating to the APS modification, and finally the Fe_3O_4@APS nanoparticles (AMNPs) were obtained (see Figure S3a in the Supporting Information). These AMNPs could easily be dispersed into aqueous solution and then gathered by magnet in 5 s (see Figure S3b in the Supporting Information). Superparamagnetism and immediate magnetic response of AMNP guaranteed the rapid magnetic separation necessary for HNPs enrichment from aqueous solutions and body fluids.

Defensin HNP 1 was prepared by Fmoc solid phase peptide synthesis, and oxidative folding was processed as being reported, then verified by analytical HPLC and MALDI-TOF-MS (Figure S4).[28] According to our result, from 1 μL of HNP 1 aqueous solution, the detection limit of HNP 1 is 40 ng. Alternatively, AMNPs could collect enough amount of HNP 1 for MALDI-TOF-MS analysis from 7.5 μL of 4 μg/mL aqueous solution, not to mention 20 μL (Figure 1a). The quantification test of HNP 1 enriched by AMNPs from 0 to 100 μg/mL demonstrated that over 87% HNP 1 was enriched, and the calibration curve was confirmed to be linear (see Figure S5a in

dx.doi.org/10.1021/am4021523 | ACS Appl. Mater. Interfaces 2013, 5, 8267−8272

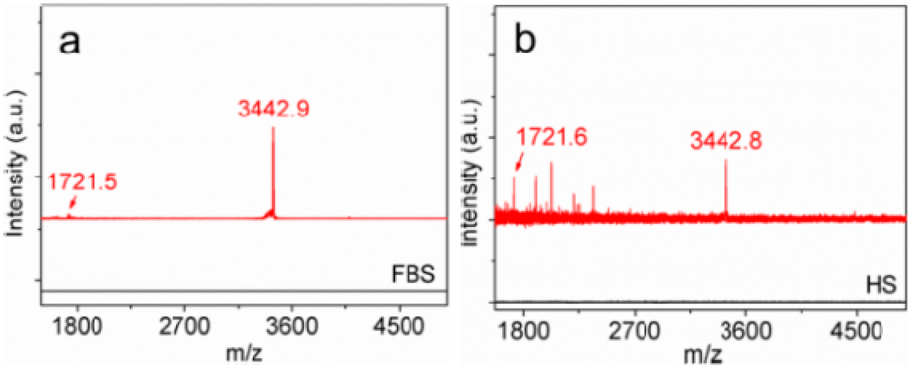


Figure 3. MALDI-TOF-MS spectra: (a) 7.5 μL of 40 μg/mL HNP 1 in FBS was treated with 1 μL AMNPs (red), HNP 1 in FBS with the concentration of 40 μg/mL (black); (b) AMNPs collected from 5 μL HS solution of HNP 1 with the concentration of 100 μg/mL (red), HNP 1 in HS with the concentration of 100 μg/mL (black).

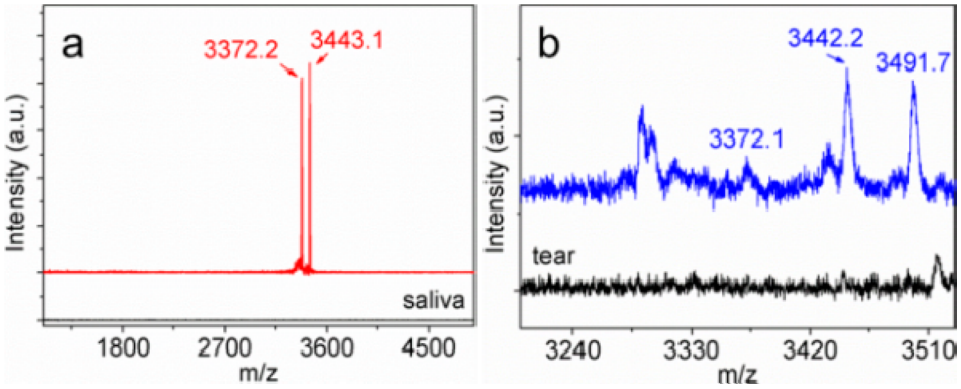


Figure 4. MALDI-TOF-MS spectra: (a) AMNP enriched HNPs from 400 μL of saliva sample without post added HNP 1 (red), saliva collected from healthy individuals without treatment (black); (b) AMNPs enriched HNPs from 280 μL of tear sample without postadded HNP 1 (blue), tear collected from healthy individuals without treatment (black).

the Supporting Information), indicating the selectivity and efficiency of AMNP for enriching HNP 1 molecules. However, in the presence of reducing reagent such as TCEP and GSH, AMNP failed to collect detectable amount of reduced HNP 1 from 40 μg/mL solution up to 25 μL (Figure 2a). Also, neither Fe_3O_4@COOH nor MNPs was able to collect detectable amount of HNP 1 from the same solution, which confirmed the successful modification of ALF onto magnetic nanoparticles (Figure 2b). These results indicated the specific recognition and binding between ALF and HNP 1 molecules.

Our studies showed that AMNP can be repeatedly deactivated and reactivated in HNP 1 collection by controlling the presence of Zn^{2+} ion in the aqueous solution (Figure 1b). The presence of Zn^{2+} ion is critical for the activation of ALF, and only the activated ALF could bind to HNP 1 with high affinity and specificity. Removal of Zn^{2+} ion from activated ALF or ALF-HNP 1 complex could readily deactivate ALF, thus trigger the subsequent HNP 1 releasing and silent ALF response toward HNP 1. Therefore, the recycle of AMNPs could be easily achieved by handling with the Zn^{2+} ion solutions. The process of collecting and releasing of HNP 1 by AMNPs is shown in Figure 1b. HNP 1 (4 μg/mL, 20 μL) was first enriched by AMNPs (activated ALF), then the chelation and removal of Zn^{2+} from ALF by EDTA inactivated the ALF and triggered nearly complete release of all the collected HNP 1 molecules from AMNPs. After that, addition of Zn^{2+} reactivated the AMNPs, restored its ability to specifically bind HNP 1, which could be readily reused in the next cycle. The whole process could be repeated 3 times (see Figure S5b in the Supporting Information).

Successful enrichment of HNP 1 from complex environments such as body fluids is crucial for practical applications of AMNPs. Sample complexity is one factor affecting the performance of AMNPs detecting HNP 1. It was confirmed that 1 μL of the prepared AMNPs with less than 0.78 fmol of ALF could enrich HNP 1 not only from fetal bovine serum (FBS) solution (Figure 3a), and from 10% diluted human serum (HS) solution (Figure 3b). However, we failed to enrich detectable HNP 1 from the same volume of human serum with the same HNP 1 concentration. Alternatively, when we increased the sample volume to 650 μL, the same amount of AMNPs was able to enrich detectable HNP 1 molecules from HS solution with the concentration of 0.8 μg/mL (see Figure S5c in the Supporting Information).

Usually, 650 μL is not an unacceptable volume for most clinical analysis of body fluid samples. The expression of HNP 1−3 can be as high as 2715 μg/mL in the saliva of oral cancer patients,[29] 170 μg/mL or even higher in the fluid of tumor tissues[30] or microenvironments, and 2.6 μg/mL in tears of patients with eye diseases,[31] which is far beyond the concentrations tested in our experiment with small volume. All these results implied the potential application of AMNP in enriching HNP 1 from bio samples and suggest that they could be directly applied in biological detection and clinic diagnosis. Saliva, one of the most common body fluids composed of more than 2000 different peptides and proteins, has been chosen as

pathological sample for analysis and diagnosis of oral disease.[32] A volume of 400 μL saliva from healthy individuals was collected and analyzed, the MALDI-TOF-MS spectra were shown in Figure 4a. The peak at 3443.1 indicated the successful collection of detectable HNP 1 from saliva; besides, there was another peak located at 3372.2, which corresponded to HNP 2.[31] Tear samples were also tested for its clinical significance in pathological diagnosis, and in our study 280 μL of tear sample was treated by AMNP. As it was shown in Figure 4b, no visible HNPs peaks were identified in the mass spectrum when tear was directly analyzed by MALDI-TOF-MS; while after AMNP enrichment, signals of HNP 1 (3442.2) and HNP 2 (3372.1) were clearly identified in the mass spectrum. Also there was an additional peak which might be assigned as HNP 3 (3491.7).[33] It was reported that, for healthy individuals, the concentration of HNP 1 in saliva was 0.8 μg/mL,[12] and in tear it was 0.17 μg/mL.[34] The detectable HNP 1 enriched in these body fluid samples confirmed the adequate binding affinity of AMNPs to HNPs, indicating the potential applications for enrichment of HNPs from clinical body fluid samples in the near future.

After we decreased the volume or diluted the tear and saliva sample, the same amount AMNPs were not able to collect detectable HNPs for MALDI-TOF-MS analysis. Therefore, there would be a proper volume range for the successful analysis of HNP 1 in pathological samples from patients. We propose, after analyzing a large number of clinical samples, AMNPs can be used for qualitative or semiquantitative analysis of HNP 1 in body fluid samples. To further quantify the exact amount of HNP 1, EDTA can be employed to release the collected HNP 1 from AMNPs for further analysis such as ESI-MS or ELISA assay.

4.. CONCLUSIONS

In conclusion, tailored AMNPs nanodetectors of HNP 1−3 were successfully designed and fabricated for the first time. They can be directly used in the analysis of biological samples, and they could be directly applied in clinical diagnosis and pathological studies at molecular level. This project can provide new insights for disease detection and therapeutic evaluation and promote the advances of biological diagnostic techniques by introducing nanotechnology and nanomaterials into traditional biological methods.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures showing TEM images Fe_3O_4 nanoparticles and Fe_3O_4@SiO_2 nanoparticles; ζ potentials of Fe_3O_4, Fe_3O_4@SiO_2, Fe_3O_4@APS, and Fe_3O_4@COOH nanoparticles and IR spectra of Fe_3O_4, Fe_3O_4@SiO_2, Fe_3O_4@APS, Fe_3O_4@COOH and Fe_3O_4@ALF nanomaterials; magnetic hysteresis curve of Fe_3O_4, Fe_3O_4@SiO_2 and Fe_3O_4@APS at 300 K; photo of AMNPs solution in the absence and presence of magnetic force; HPLC spectrum and MALDI-TOF-MS spectrum of HNP 1; the calibration curve of HNP 1 from 0 to 100 μg/mL enriched by AMNPs; MALDI-TOF-MS spectra of AMNPs treated by EDTA, Zn^{2+} and HNP 1, and MALDI-TOF-MS spectrum of HNP 1 collected from 650 μL HS solution with concentration of 0.8 μg/mL. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was financially supported in part by grants from the State High-Tech Development Plan (2012AA020804), National Natural Science Foundation of China project (81201194, 30970784, 31170873, and 81171455), General Financial Grant from the China Postdoctoral Science Foundation (2012M520219), National Key Basic Research Program of China (2009CB930200), National Distinguished Young Scholars Grant (31225009) from the National Natural Science Foundation of China and Chinese Academy of Sciences (CAS) "Hundred Talents Program" (07165111ZX).

■ ABBREVIATIONS

HNP, human neutrophil peptide
MNP, magnetic nanoparticle
ALF, anthrax lethal factor
AMNP, anthrax lethal factor modified magnetic nanoparticles
TEOS, tetraethylothosilicate
APS, (3-aminopropyl)triethoxysilane
EDC, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
NHS, N-hydroxysuccinimide
HS, human serum
FBS, fetal bovine serum

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