Selective enrichment and sensitive detection of candidate disease biomarker using a novel surfactant-coated magnetic nanoparticles

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Abstract. In this study, novel surfactant-coated magnetic nanoparticles were synthesized and evaluated for enrichment performance towards the sensitive detection of disease biomarkers. Surfactants with phosphate ester groups (RD35A and RD66) were used as a coating to reduce aggregation and to enhance the nanoparticle dispersion. Importantly, sensitive enrichment of the target proteins using the antibody-functionalized magnetic nanoparticles (Ab@MNP) was obtained, with a five-fold increase in recovery compared to uncoated magnetic nanoparticles. Similarly, phosphopeptide enrichment using the NTA@MNP in standard samples showed that the nanoparticles could selectively enrich phosphorylated peptides.

1. Introduction

Within the past few decades, synthesis and application of various nanomaterials is actively explored, as these materials are implicated in several fields which include electronics, bio (chemical) sensing and disease biomarker screening and detection [1-5]. Among all of the diverse classes of nanomaterials, magnetic nanoparticles received considerable attention due to its remarkable properties such as magnetic property, biocompatibility and excellent surface functionality which attracted a growing significance of these materials in various applications especially in biomedicine and bioengineering [1]. In spite of its unique and immeasurable applicability, there is still a great deal of interest to investigate new surface protection strategies to enhance the nanoparticle dispersion and to understand the behaviour of these nanomaterials, particularly the aggregation behaviour which influences its performance towards bioanalytical applications.
Aggregation phenomenon of magnetic nanoparticles is one of the crucial factors which can affect the performance for their desired application [6]; hence, alters its analytical performance and detection sensitivity. Commonly, attractive magnetic forces between particles draw the particles to come close in proximity to each other and result in aggregation [7]. Thus, protection of surface of the nanoparticle is of great importance in order to minimize the attractive forces between particles. A range of protection strategies were reported, including metal inorganic coating [8], organic/functional group [9] and polymer/surfactant coating [10-11]. Among all of these, surface protection using surfactants were extensively investigated because of its suitability and ease in providing active surface functionality towards different target ligands.

This paper describes the use of new surfactants with phosphate ester groups as a protective layer in the surface of the nanoparticles. To the best of our knowledge, this is the first report which utilizes this unique class of surfactants which minimizes magnetic attractive forces by enhancing electrostatic repulsion with optimized amphiphilicity of the resulting particles. Preliminary results showed that a significant improvement in the nanoparticle dispersion, though an in-depth investigation on the effect of these surfactants to the aggregation behaviour of the nanoparticles is still underway. Likewise, initial application performance of these surfactant-stabilized nanoparticles was demonstrated by fabricating an efficient nanoprobe for the target disease biomarker enrichment and detection. Similarly, Fe-IMAC (Immobilized metal ion affinity chromatography) nanoprobe was constructed using the Nitrilotriacetic acid (NTA)-functionalized MNP for the selective phosphopeptide enrichment.

2. Experimental
All chemicals and reagents were used as received without further purification unless otherwise stated. FeCl2·4H2O (≥ 99%), HCl (≥37%), NaOH (≥ 98%), and NH4OH (25%) were obtained from Sigma-Aldrich; FeCl3, and Suberic acid bis(N-hydroxy-succinimide ester (DSS, ≥ 95%) were obtained from Sigma; Aminopropyltrimethoxysilane (APS, ≥ 97%), ethanolamine (≥ 99.5%) and N-β-N-β-bis(carboxymethyl)-L-lysine (NTA-Lysine, ≥ 97%) from Aldrich; 1-propanol (≥ 99%) and tetraethyl orthosilicate (TEOS, ≥ 98%) from Acros Organics; dimethyl sulfoxide (DMSO) from Thermo Scientific, and Tris (99.8 – 100%) from PlusOne. Surfactants (RD35A and RD66) were supplied by En Hou Polymer Chemical Ind. Co., Ltd. (Taiwan) and amine methoxyethylene glycol (MEG) was provided by Chun-Cheng Lin of National Tsing Hua University. Standard alpha-fetoprotein (AFP) was obtained from Abnova and Anti-AFP antibody was obtained from Thermo Scientific. α-casein and β-casein were purchased from Sigma.

2.1 Synthesis of magnetic nanoparticles (MNPs) and fabrication of nanoprobe
Core magnetic nanoparticles (MNP) and amine-functionalized MNP (N009) were synthesized as adopted from [12]. Similarly, Ab@MNP and NTA@MNP were fabricated adopted from our previous works [5] and [13], respectively with slight modifications. For the surfactant coating, to a solution of 1-propanol (80%), the required amount of surfactants (RD35A and RD66) were added and sonicated for 30 minutes. In a separate flask, the previously prepared core magnetic nanoparticles (50 mg) were dissolved in 25% 1-propanol solution (10 mL) and were sonicated for 30 minutes. The two mixtures were then mixed and stirred vigorously for 4 hours at room temperature. The mixture was transferred to a pre-heated silicon bath (~55 °C) and NH4OH (20.8 mmol) and TEOS (1 mmol) was added sequentially and continuously stirred for another 4 hours. APS (1 mmol) was then added and incubated for overnight. The particles were then isolated by centrifugation, washed with 1-propanol three times, then with water three times, and the residue dried in vacuum and stored for further use. Two different batches of the surfactant-stabilized NH2@MNP (with different amount of surfactants) were synthesized, 11-NH2@MNP and 5-NH2@MNP for Batch 1 and Batch 2, respectively.
2.2 Protein and phosphopeptide Enrichment
Antibody@MNP (Ab@MNP, 2 μL, 10 mg/mL) were added to a standard protein solution in phosphate buffered saline (PBS, pH ~ 7.4, 1-5 ng/mL). The solution was incubated at room temperature for 1 hour in a rotary mixer. After extraction, the MNP were separated using a magnet, washed twice with Tween-TBS (TTBS, 200 μL) and then twice with deionized water (200 μL). For LOD determinations, AFP was detected by liquid chromatography-mass analysis using multiple reaction monitoring (MRM) mode. For % recovery experiments, SDS-PAGE followed by silver staining was carried out. Similarly, phosphopeptide enrichment was carried out using the Fe-IMAC method and Ni-NTA beads as described in [13]. Different loading buffers were prepared and 0.5 μg α, β-casein was used to demonstrate the applicability of the NTA@MNP for the enrichment of phosphopeptides by MALDI-TOF analysis.

3. Results and Discussion
3.1 Physical properties and surface morphology of core and functionalized nanoparticles
The size and morphology of the core and functionalized magnetic nanoparticles (MNP) were investigated by TEM (Figure 1). TEM images revealed that the synthesized surfactant-coated MNP had a core-shell structure with an average core diameter of 21 nm. Although an aggregate of iron oxide nanoparticles per shell was noticeable, production of a stable core-shell morphology was proven. Moreover, the images also show that an empty shell, probably an empty silica particle formed after silica coating, was observed. The production of empty silica nanoparticles is reportedly difficult to prevent completely especially during silica coating process [14]. Furthermore, preliminary results showed that the rate of sedimentation or the decay time (data not shown) of the core and the uncoated-NH2@MNP (N009) were shorter compared to the surfactant-stabilized magnetic nanoparticles. This suggests that the new surfactants can help improve the dispersity of the nanoparticles in an aqueous environment.

Figure 1. TEM images of magnetic nanoparticles (a) core MNPs and (b) N009 NH2@MNP (c) RD66 and (d) RD35A

Figure 2. XRD pattern of (a) core MNPs and (b) N009 NH2@MNP (c) RD66 and (d) RD35A

The crystal structure of the nanoparticles was monitored using X-ray diffraction (XRD) analysis as shown in Figure 2. XRD data revealed that even after surface modification of the nanoparticles, its crystal structure was not altered. Indeed, the obtained XRD peaks were still consistent with that of the standard Fe3O4 diffraction pattern. Importantly, the amorphous nature of the nanoparticle after surfactant and silica coating can be inferred based on the XRD pattern as shown in Figure 2c and d and is also consistent with the TEM result which confirms the presence of silica shell structure. On the
other hand, FT-IR and EDX spectra of the core and functionalized magnetic nanoparticles are shown in Figure 3 and 4, respectively. Characteristic peaks for the Si-O and NH band were observed in the functionalized nanoparticles which were not observed in the core MNP’s. Moreover, elemental analysis using EDX showed that a higher Si peak was noted in all of the functionalized nanoparticles which are consistent with the IR results.

![Figure 3. IR spectra of (a) core MNPs and NH$_2$@MNP (b) N009 (c) RD66 and (d) RD35A](image)

![Figure 4. EDX spectra of (a) core MNPs and NH$_2$@MNP (b) N009 (c) RD66 and (d) RD35A](image)

**3.2 Nanoprobe-based enrichment and detection application**

(a) **Alpha-fetoprotein (AFP) enrichment and detection**

To demonstrate the potential application of the newly synthesized surfactant-stabilized nanoparticles towards biomarker enrichment and detection, an antibody-conjugated nanoprobe was fabricated. Alpha-fetoprotein (AFP) was chosen as a model protein since this is the widely known biomarker for hepatocellular carcinoma. Three different batches of AFP-based nanoprobes were designed which includes the Ab@RD66, Ab@RD35A and Ab@N009. To evaluate the specificity of the fabricated nanoprobes, enrichment was conducted in a mixture of proteins (AFP, Lactoferrin and myoglobin). The results showed (Figure 5) that the percentage recovery of the nanoprobes based on the surfactant-stabilized nanoparticles were exceptionally higher compared to the nanoprobe without surfactant. As shown, about 5.5-fold enhancement and 3-fold enhancement for Batch 2 and Batch 1, respectively, was noted for the surfactant-stabilized nanoprobes compared to that of uncoated nanoparticles (N009). Further, it is noticeable that the said enrichment enhancement is not correlated to the antibody density (shown in Figure 6); instead other factors may contribute for the observed enhancement. Plausibly, the dispersity of the nanoparticles may play an important role in the observed performance of the surfactant-stabilized nanoprobes towards the target protein enrichment. Hence, an experiment which focuses on understanding the aggregation nature of these nanoprobes and relating it in its enrichment performance is underway.
get rid of the non-specific binding of healthy controls (20 ng/mL) [15]. Norm buffers and Fe$^{3+}$-metal activation.

(b) Phosphopeptide enrichment

Furthermore, multiple reaction monitoring (MRM) analysis was conducted to evaluate the limit of detection (LOD) of these nanoprobes. All data were processed in MultiQuant software (V 2.1, AB Sciex). The most intense transition was used for peak area integration while the other 5 transitions were used to confirm the protein identity. Results showed that the surfactant-stabilized nanoprobes, especially the one coated with RD66 surfactant, gave the best performance among all other nanoprobes fabricated. The obtained LOD for the RD66-coated nanprobe was able to detect to as low as 0.5 ng/mL (7.3 pM) with excellent S/N ratio (128), which is far lower than the AFP concentration of healthy controls (20 ng/mL) [15]. Further condition optimization and validation is currently being conducted to completely demonstrate the general applicability of these nanoprobes towards screening and detection of different disease biomarkers.

(b) Phosphopeptide enrichment

As a proof-of-concept, NTA-functionalized magnetic nanoparticles were fabricated specifically for phosphopeptide enrichment. Standard α- and β-casein (0.5 μg) sample and 12 μg of the NTA nanrobe were used in the enrichment process following the sequential elution with the loading buffers and Fe$^{3+}$-metal activation. It is noteworthy, moreover, that the newly-fabricated nanoprobes were unblocked nanoprobes, which means that no blocking agent was added as compared to some of the reported literatures [13]. Normally, conjugation of blocking agent to the nanprobe was made to get rid of the non-specific binding to other analytes in the mixture. However, the result shows that selective enrichment of the target phosphopeptides were still detected even the use of the unblocked NTA nanprobe (as shown in Figure 8). However, in order to improve the selectivity and enrichment
efficiency, further condition optimization, particularly of the loading buffer, nanoprobe amount and enrichment protocol should be further investigated. Lastly, fabrication of NTA-nanprobe with the optimized conjugation of the desired blocking agent is in progress.

![Figure 7. Representative MALDI spectra of mixture of α- and β-casein purified from Fe-NTA nanoprobe (a) RD35A-2 (b) RD66-2 (c) RD35A-1 and (d) RD66-1 to enrich phosphopeptide](image)

### 4. Conclusion

It has been demonstrated that RD35A and RD66 surfactants successfully protects the surface of the iron oxide magnetic nanoparticles. Characterization revealed that surfactant coating did not alter the surface morphology and properties of the nanoparticles. Most importantly, it has been shown the potential application of the nanomaterials as efficient nanoprobes for both target protein disease biomarker and phosphopeptide enrichment and detection. Although demonstration of the potential application of these nanoprobes has been illustrated successfully, detailed and in-depth study for validation in the enrichment and detection aspect is necessary and is currently being investigated. Understanding the effect of these surfactants to the nature of aggregation of magnetic nanoparticles is likewise in progress.

### 5. References

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