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ARTICLE

Aluminium glycinate functionalized silica nanoparticles for highly specific separation of phosphoproteins†

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Selective separation of intact phosphoproteins from complex biological samples is essential for the ongoing top-down phosphoproteomics, but challenges still remain. Herein, aluminium hydroxide functionalized silica nanoparticles (denoted as AGNP) was synthesized by a facile approach and applied for specific capture of phosphoproteins. The selectivity and binding capacity of AGNP were evaluated by using caseins (α -casein and β -casein) as phosphoproteins and nonphosphoproteins (bovine haemoglobin, bovine serum albumin, horseradish peroxidase, myoglobin and lysozyme) as nonphosphoproteins. The results indicated that the AGNP showed high binding capacity and selectivity for phosphoproteins (α -casein 1190 mg g⁻¹ and β -casein 1060 mg g⁻¹). In addition, the AGNP can be used to selectively capture and enrich phosphoproteins from protein mixture and drinking milk samples. The good results demonstrate the potential of the AGNP in phosphoproteomics analysis.

Introduction

Phosphorylation, as one of the most common post-translational modifications (PTMs) of proteins, plays critical roles in regulating biological functions.^{1, 2} The abnormal level of phosphoproteins are implicated in several human diseases, such as Alzheimer's disease and Parkinson's disease.^{3, 4} Therefore, detailed analysis of phosphoproteins is critical for understanding these disease mechanisms and biological processes. However, direct mass spectrometry (MS) analysis of protein phosphorylation is still challenging due to the extreme complexity of biological samples and low stoichiometry of protein phosphorylation.

Currently, selective enrichment of phosphopeptides from complicated protein digests prior to MS analysis has become an effective way to enhance the MS analysis results.⁵⁻¹² Nevertheless, this bottom-up proteomics also suffers from drawbacks such as loss of labile PTMs and sequence variants.¹³ In contrast, top-down MS analysis of intact phosphoproteins could provide a better description of protein phosphorylation^{14, 15} and regain considerable attention with new MS instruments such as Fourier transform ion cyclotron resonance^{16, 17} and Orbitrap MS^{18, 19}. However, there are still few studies devoted to the separation and enrichment of intact phosphoproteins.²⁰⁻²³

The common methods for phosphopeptide enrichment are

immobilized metal affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC), but they are rarely reported to separate phosphoproteins mainly because of the low adsorption efficiency.^{20, 24} Very recently, Hwang *et al.* reported the synthesis of multivalent ligand functionalized nanoparticles for specific enrichment of phosphoproteins.²³ However, this method need to pre-synthesize affinity ligand using a multi-step reaction using harsh conditions. Therefore, developing a facile and more effective approach for separation of intact phosphoproteins is urgently needed.

Herein, aluminium glycinate functionalized silica nanoparticles (AGNP) were synthesized for the selective capturing of phosphoproteins. Aluminium glycinate is an antacid with good hydrophilicity, and was exploited as a kind of novel IMAC ligand for the first time in this work. Moreover, the nanoscale AGNP (<100 nm) should perform better than IMAC or MOAC-based microspheres because their good solubility and high surface/volume ratio would result in higher binding capacity. The properties of AGNP prepared by a facile method were characterized in detail. The adsorption behaviours of AGNP for intact phosphorylation proteins were investigated. In addition, the practicability of AGNP for biological applications were evaluated by isolation of intact phosphoproteins from diluted drinking milk.

Experimental

Materials and reagents

Tetraethoxysilane (TEOS), 3-(N-morpholino) propanesulfonic acid (MOPS), sodium cyanoborohydride (NaCNBH₃, 95%), trifluoroacetic acid (TFA) and α -Al₂O₃ (99.99% metals basis, 30 nm) were purchased from Aladdin Chemistry Co., Ltd

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(Shanghai, China). 3-Aminopropyl triethoxysilane (APTES) was purchased from Chemical Factory of Wuhan University (Wuhan, China). Aluminium glycinate (97%) was purchased from TCI (Shanghai, China). Ammonium hydroxide (25%), acetonitrile (HPLC grade), glutaraldehyde (GA, 25%), anhydrous ethanol and isopropanol were all obtained from Sinopharm Chemical Reagent, Co., Ltd. (Shanghai, China). α / β -casein from bovine milk and myoglobin (Mb) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine hemoglobin (BHb), bovine serum albumin (BSA), lysozyme from chicken egg white (Lyz) and horse-radish peroxidase (HRP) were obtained from Shanghai Lanji Co. Ltd. (Shanghai, China). BeyoAP alkaline phosphatase (1 U/ μ L) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). All reagents listed above were of analytical grade or better. Deionized water (18.2 M Ω cm⁻¹) was prepared with a Milli-Q water purification system (Millipore, USA). Drinking milk was purchased from local supermarket.

Synthesis of SiO₂ nanoparticles

Monodisperse silica nanoparticles were synthesized according to our previous work²⁵ with minor modification. In brief, 6 mL of TEOS was added to the mixture of 100 mL of ethanol, 4 mL of deionized water and 4 mL of 25% ammonium hydroxide solution with vigorous stirring at 30 °C and the reaction was continued for 24 h. Subsequently, the resultant silica particles were washed with ethanol and dried at 60 °C under vacuum for further use.

Synthesis of AGNP

500 mg of silica nanoparticles were dispersed in a mixture of isopropanol (50 mL) and APTES (1 mL) by ultrasonication and heated to 85 °C for 2 h to obtain the amino-functionalized silica nanoparticles (denoted as SiO₂-NH₂).²⁶ The product was washed with ethanol and dried at 60 °C under vacuum for further use.

The SiO₂-NH₂ nanoparticles were dispersed in 50 mL of MOPS buffer solution (pH 7.4, 10 mM) containing 2% glutaraldehyde. Then 200 mg of NaCNBH₃ was added and stirred for 6 h. After the reaction, the product (SiO₂-CHO) was washed several times with deionized water.

The SiO₂-CHO nanoparticles were re-dispersed in 50 mL of MOPS buffer solution (pH 7.4, 10 mM). Then, 500 mg of aluminium glycinate was added to above mixture. After stirring the mixture for 4 h, the product (AGNP) was washed as SiO₂-CHO and dispersed in water for further use. All the reactions were performed at 25 °C.

Dephosphorylation measurements

α / β -casein were dissolved at concentration of 2 mg mL⁻¹ in a 100 mM Tris-HCl buffer containing 1 mM MgCl₂ (pH 8.0). The sample solutions were incubated in a water bath at 37 °C, and then alkaline phosphatase (2 U) was added. After incubation for 2 h, all the dephosphorylation of proteins were transferred to a water bath at 75 °C for 5 min. Then the samples (noted as Dep- α -casein and Dep- β -casein, respectively) were cooled down to room temperature for further use.

Protein adsorption properties of AGNP

The adsorption experiments were conducted in a centrifuge tube at room temperature. All sample solution were prepared in binding buffer (pH 6.0, 10 mM MOPS buffer solution containing 0.2 M NaCl). In the isothermal adsorption experiments, 0.50 mg (for phosphoproteins) and 1 mg (for nonphosphoproteins) of AGNP were vortex-mixed with 1 mL of different proteins at different concentrations ranging from 0.10 to 0.80 mg mL⁻¹ for 4 h. Then the nanoparticles were centrifuged at 14000 rpm for 10 min and the protein concentration in the supernatant was determined with a UV-Vis spectrometer.

The equilibrium adsorption capacity (Q , mg g⁻¹) was calculated according to

$$Q = \frac{(C_0 - C_e)V}{m} \quad (1)$$

where C_0 is the initial protein concentration (mg mL⁻¹), C_e is the supernatant protein concentration (mg mL⁻¹), V is the volume of protein solution (mL) and m is the weight of AGNP (g). The adsorption isotherms were fitted by the Langmuir model (eqn (2)),

$$\frac{C_e}{Q} = \frac{C_e}{Q_m} + \frac{1}{K_L Q_m} \quad (2)$$

where K_L is the Langmuir constant that directly relates to the adsorption affinity (L mg⁻¹) and Q_m is the saturated capacity (mg g⁻¹). Q_m and K_L can be calculated by plotting C_e/Q as a function of C_e .

In the kinetic binding experiments, 0.50 mg (for phosphoproteins) or 1 mg (for nonphosphoproteins) of AGNP was incubated with 1 mL of protein solution for different incubation times ranging from 15 to 240 min.

Selective capture of phosphoproteins from standard protein mixtures using AGNP

In the selective adsorption experiments, 0.50 mg of AGNP was incubated with 1 mL of a mixed solution of different mass ratio of BHb/ β -casein in binding buffer for 4 h.

0.50 mg of AGNP was incubated with 1 mL of a mixed solution of 0.05 mg mL⁻¹ Lyz, 0.70 mg mL⁻¹ BSA and 0.40 mg mL⁻¹ α -casein in binding buffer for 4 h. After centrifugation, the supernatants were collected. Then the AGNP-protein conjugates were rinsed with binding buffer once. The captured species were eluted with 10% (v/v) ammonium hydroxide. The supernatants and the eluate was analyzed by HPLC.

0.5 mg of AGNP was added into MOPS buffer (500 μ L, pH 6.0, 10 mM + 0.2 M NaCl) containing a protein mixture (0.6 mg mL⁻¹ BSA and 0.6 mg mL⁻¹ β -casein), and incubated at room temperature for 4 h. After that, AGNP with captured phosphoprotein were separated from the mixed solution by centrifugation. After twice washing with 50% ACN containing 1% TFA (500 μ L) to remove the non-specifically adsorbed proteins, the trapped phosphoproteins were finally eluted with ammonium hydroxide (10%, 200 μ L). The protein solutions in each step (including the stock, supernatant, wash and elute

solutions) were all collected by centrifugation and used for SDS-PAGE analysis.

Selective separation of phosphoproteins from drinking milk using AGNP

200 μL of drinking milk was dissolved in 1800 μL of binding buffer. Then 2 mg AGNP were added into 1 mL of 10-fold diluted milk solution and incubated at room temperature for 4 h. After washing with loading buffer (500 μL) to remove the non-specifically adsorbed proteins, the trapped phosphoproteins were finally eluted with ammonium hydroxide (10%, 1 mL). The diluted milk sample, supernatant, and eluent were collected and analyzed by SDS-PAGE.

Reusability of AGNP toward phosphoprotein

To estimate the reusability of AGNP, 2 mg of AGNP was incubated with 1.5 mL of α -casein solution (1.6 mg mL^{-1}) for 4 h at room temperature. After washing with 0.5 M ammonium hydroxide and 10 mM HCl, the recovered AGNP was reused for protein adsorption.

Characterizations

Transmission electron microscopy (TEM) and energy dispersive X-ray spectrometry (EDS) were performed on a FEI Tecnai G2 F20 (FEI, USA) at 200 kV. The Brunauer-Emmett-Teller (BET) specific surface areas of products were measured using an ASAP 2020 (Micromeritics, USA). Fourier-transform infrared (FT-IR) spectra were taken on a Nicolet 6700 spectrometer (Thermo Fisher, USA) using KBr pellets. Thermogravimetric analysis (TGA) was performed for power samples with a heating rate of $10\text{ }^\circ\text{C min}^{-1}$ using a Netzsch STA 409 (Germany) thermogravimetric analyzer under nitrogen atmosphere up to $800\text{ }^\circ\text{C}$. The X-ray photoelectron spectroscopy (XPS) analysis experiment was determined using an XPS Thermo ESCA-LAB 250XI (Thermo Fisher, USA). The zeta potentials of nanoparticles were observed using a Malvern Zetasizer Nano ZS. The data of adsorption were obtained using a UV-1950 spectrophotometer (Beijing, China). Other chromatographic measurements were performed using a Shimadzu Prominence LC-20A series HPLC (Kyoto, Japan) and an Agela Technologies Venusil XBP C8 (100 mm \times 4.6 mm, 5 μm , 300 \AA) column (Tianjin, China). The proteins were eluted with a linear gradient from 3% to 60% buffer B (buffer A, 0.1% TFA in water; buffer B, 0.08% TFA in ACN) over 8 min at a flow rate of 1.5 mL min^{-1} at $40\text{ }^\circ\text{C}$. The injected sample volume was 20 μL , and the samples were detected using a UV detector at 280 nm. Quantitative analyses analysis of the protein solutions were performed from a linear calibration curve of peak area versus concentration. Electrophoresis of proteins was performed using regular SDS-PAGE (Bio-Rad, CA, USA). Proteins were stained with Coomassie Brilliant Blue R-250.

Results and discussion

Preparation and characterization of AGNP

The aluminium glycinate was immobilized on silica nanoparticles through a step by step process. The general scheme for preparation of AGNP is illustrated in Fig. 1. Firstly, silica nanoparticles were synthesized through the Stober method²⁷, then surface-modified with a monolayer of the coupling agent APTES by heating the mixture in isopropanol at $85\text{ }^\circ\text{C}$. The amino-functionalized silica subsequently reacted with glutaraldehyde to furnish the aldehyde-modified silica nanoparticles. Finally, AGNP was prepared by reaction between $\text{SiO}_2\text{-CHO}$ and aluminium glycinate.

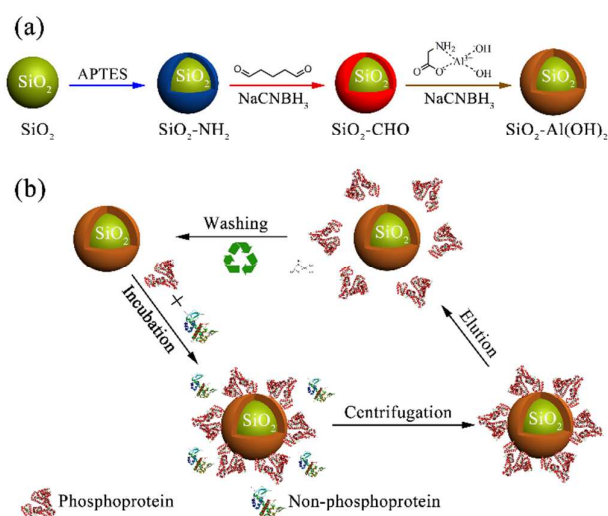


Fig. 1 Preparation of AGNP and its application for specific separation of phosphoproteins.

TEM images of SiO_2 and AGNP are shown in Fig. 2(a-d). It was observed from Fig. 2a and b that the SiO_2 nanoparticles have an average diameter of 75 nm. After gradually modified, the diameter of the AGNP increased to *ca.* 80 nm, corresponding to a 5 nm thick modified layer.

The composition of the resulting AGNP was observed by EDS. As shown in Table S1 (ESI[†]), C, O, Si and Al were main elements found, corresponding to XPS result as shown in Fig. S1 (ESI[†]). It was indicated that aluminium glycinate was successfully modified onto the surface of silica nanoparticles.

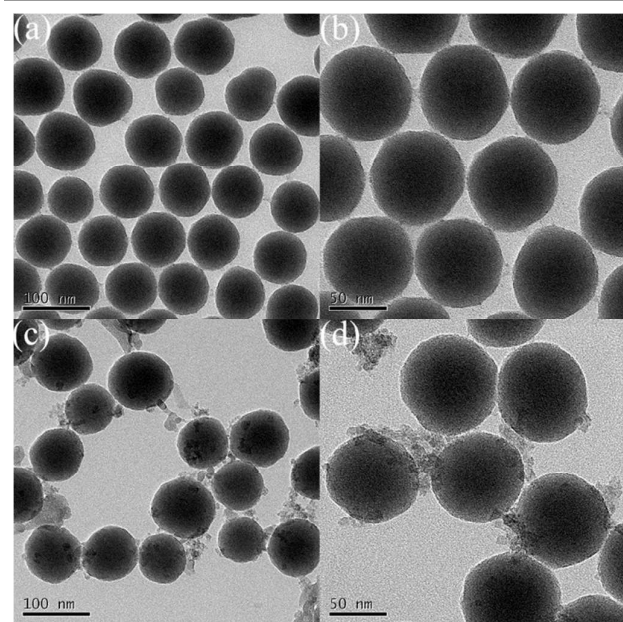


Fig. 2 TEM images of SiO₂ (a, b) and AGNP (c, d).

To further confirm the immobilization of aluminium glycinate, FT-IR spectra of the SiO₂, SiO₂-NH₂, SiO₂-CHO and AGNP were employed (Fig.3). The strong peaks at 1100 and 796 cm⁻¹ in curve a-d were due to Si-O-Si vibration of silica. The peaks near 2925 cm⁻¹ in the spectrum of SiO₂-NH₂ (curve b) was assigned to C-H adsorption, implying the existence of APTES. The characteristic band at 1715 cm⁻¹ is attributed to the C=O bond from the aldehyde group (curve c). The spectrum of AGNP (curve d) showed that the complete disappearance of the band at 1715 cm⁻¹ and the band at 1331 cm⁻¹ was raised. In addition, the bands at 752 cm⁻¹, 670 cm⁻¹, 574 cm⁻¹ were appeared in curved to Al-O stretching vibrations, suggesting that aluminium glycinate was successfully modified onto the surface of silica nanoparticles.

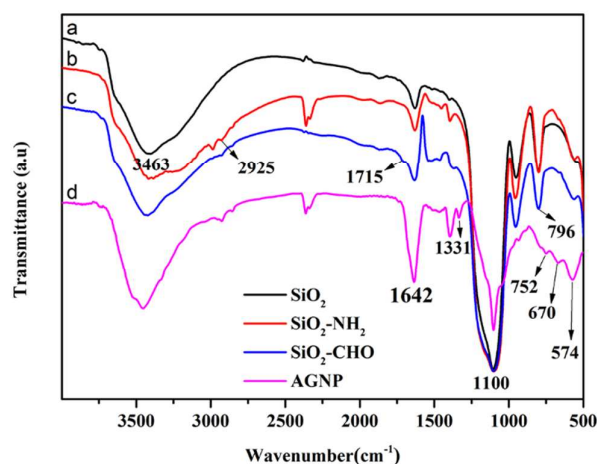


Fig. 3 FT-IR spectra of silica nanoparticles with different functional groups a) SiO₂, b) SiO₂-NH₂, c) SiO₂-CHO and d) AGNP

TGA was executed to quantitatively determine the composition of the composite nanomaterials. As shown in Fig. 4, AGNP displayed a distinct mass-loss profile above 200 °C compared to that obtained in the naked silica core (cure a). It was deduced that SiO₂-CHO (curve b) revealed a slightly high organic mass release of about 2% between 400 °C and 600 °C. The weight loss could be responsible for the weight of APTES and glutaraldehyde. Compared to curve b, the 18 wt% loss of AGNP (curve c) could be attributed to the organic component of aluminium glycinate.

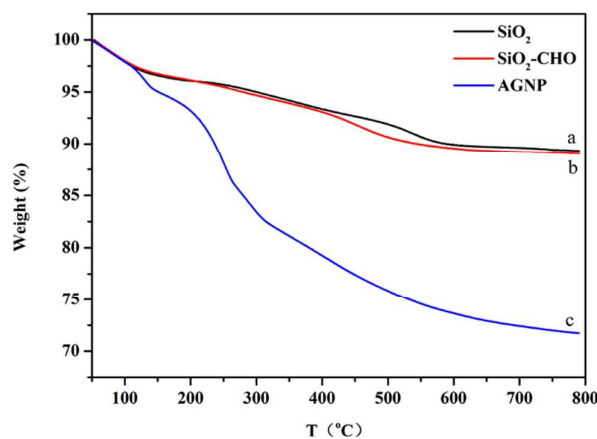


Fig. 4 TGA curves of: a) SiO₂, b) SiO₂-CHO and c) AGNP

Binding properties of AGNP

To explore the adsorption performances influenced by pH, a series of adsorption experiments with single component protein (α -casein, β -casein, BSA, and HRP) under different pH solution were investigated. Fig. S2 (ESI[†]) shows that the effect of media pH on protein adsorption capacity of AGNP is a pH-dependent process. The maximum adsorption capacity was found at pH 6.0. At a high pH range, hydroxide ions in buffer systems can form complexes with aluminium hydroxide group on the surface of AGNP. While at a low pH such as 4.0 or 5.0, the phosphoproteins were precipitated for their isoelectric point (pI) was in the range from 4.6 to 5.1. Therefore 6.0 was selected as the optimized pH.

The effect of ionic strength on phosphoproteins and nonphosphoproteins were investigated, and the results are shown in Fig. S3 (ESI[†]). It is clear that phosphoprotein adsorption capacity slowly decreased with the increment of NaCl concentration. When NaCl concentration increased up to 0.2 M, less than 4% reduction of phosphoprotein adsorption capacity was observed, whereas about 42% reduction took place on BSA and 40 % for HRP. This can be explained by the weaker nonspecific interaction (such as electrostatic interactions, covalent interactions and Van der Waals forces) between AGNP and nonphosphoprotein in the adsorption media with higher salt concentration while a stronger specific effect on phosphoproteins. The result is in agreement with the reference.²⁸ As NaCl concentration increased from 0.2 to 0.5 M, the adsorption of proteins got a balance. Therefore, 0.2 M NaCl was selected as the optimized ionic strength.

To verify the extent of specific binding with phosphoproteins (α -casein and β -casein) versus nonspecific binding with nonphosphoproteins (BSA, HRP) on AGNP. The adsorption capacity of these proteins is shown in Fig. 5a. It was observed that AGNP showed a higher adsorption capacity for phosphoproteins than nonphosphoproteins. The saturated adsorption capacities of α -casein and β -casein were 1190 mg g^{-1} and 1060 mg g^{-1} , remarkably high than those obtained for BSA and HRP, which were of 120 mg mL^{-1} and 34 mg mL^{-1} , respectively. The binding capacity for β -casein is more than 17-fold higher than the reported literature (59.6 mg mL^{-1}). Although the nonspecific adsorption of BSA is 8.5-fold higher than the previous literature,²¹ it is quite clear that AGNP in this work owns a better specific adsorptive property. And the excellent phosphorylated protein adsorption property of AGNP may be attributed to the large specific surface area ($137.8 \text{ m}^2 \text{ g}^{-1}$, Table S2 in ESI†) and affinity-like chromatography between aluminium hydroxide and phosphate group.²⁹ In addition, the adsorption isotherms were fitted well to the Langmuir model (Table 1 and Fig. S4 in ESI†).

Adsorption kinetics. Fig. 5b illustrates the adsorption kinetics of saturated protein solution onto AGNP. From the time course of binding, the adsorption amount of phosphoproteins increased significantly in the first 120 min, while nonphosphoproteins showed a horizontal tendency. The results could be explained by the fact that the phosphoproteins were facilitated to react with aluminium hydroxide based on the specifically covalent binding at the beginning. With the saturation of binding sites, phosphoproteins began to nonspecifically diffuse onto the surface of AGNP like nonphosphoproteins.

Table 1 A summary of the fitted parameters of the protein adsorption equilibrium on the AGNP

protein	Mw (KDa)	pI	Langmuir model		
			$Q_m(\text{mg g}^{-1})$	$K_L(\text{mL mg}^{-1})$	R^2
α -casein	24	4.6-5.1	1217	19.97	0.9971
β -casein	24	4.6-5.1	1191	48.64	0.8842
BSA	66.7	4.7	128.2	18.78	0.9960
HRP	44	7.2	32.57	73.52	0.9952

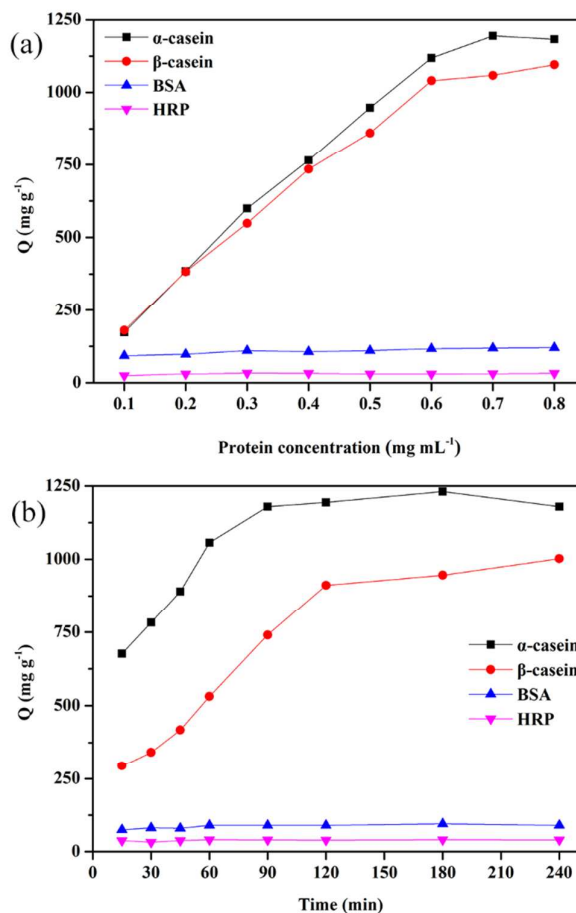


Fig. 5 (a) Adsorption isotherms of proteins onto AGNP; (b) Adsorption kinetics of proteins onto AGNP.

Nonspecific adsorption of AGNP

The resulting materials have indicated excellent binding capacity for capture phosphoproteins, however, nonspecific adsorption is also an important factor for AGNP. Five nonphosphoproteins (including BHb, BSA, HRP, Mb, Lyz) with different molecule size and pI were chosen as competitors. The result showed in Fig. 6 indicated the nonspecific adsorption capacities (BHb 107.5 mg g^{-1} , BSA 106.3 mg g^{-1} , HRP 34.5 mg g^{-1} , Mb 23.7 mg g^{-1} , Lyz 3.9 mg g^{-1}) were 10-fold lower than the specific adsorption toward phosphoproteins (α -casein 1190 mg g^{-1} , β -casein 1060 mg g^{-1}).

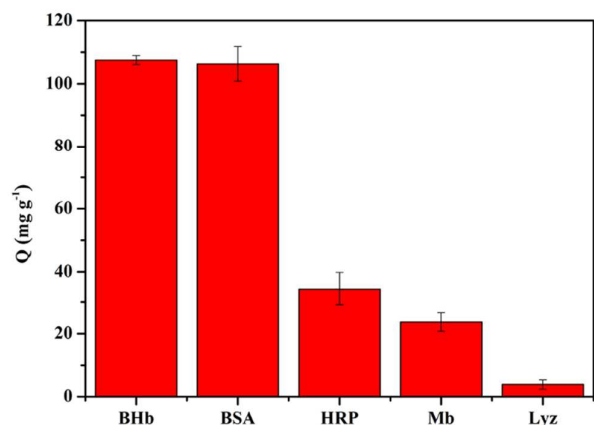


Fig. 6 Recognition of AGNP toward different nonphosphoproteins. Error bars represent the standard deviations, $n=3$.

Phosphoproteins and dephosphoproteins adsorption

To evaluate the selectivity of AGNP for phosphoproteins, α -casein, β -casein, Dep- α -casein and Dep- β -casein were chosen as model proteins. α/β -casein were two kinds of well-known phosphoproteins. While after treatment with alkaline phosphatase, there were less or no surface-exposed phosphate groups. As expected in Fig. 7, the maximum adsorption capacity of AGNP toward Dep- α/β -casein were 336.7 mg mL⁻¹ and 313.9 mg mL⁻¹, which is much lower than the adsorption capacity of α/β -casein (α -casein 1190 mg g⁻¹, β -casein 1060 mg g⁻¹). It is clear that AGNP had an excellent specific interactions with phosphoproteins.

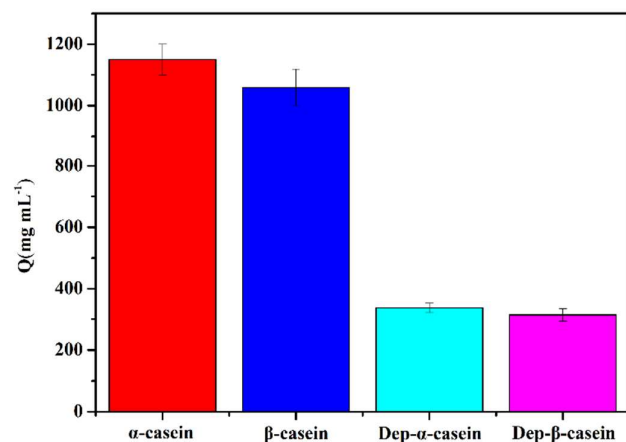


Fig. 7 Phosphoproteins and dephosphoproteins adsorption of AGNP toward α/β -casein and Dep- α/β -casein. Error bars represent the standard deviations, $n=3$.

Static adsorption of phosphoproteins

α -casein and β -casein were selected as model phosphoproteins to examine the feasibility of the AGNP for specific phosphoprotein adsorption. As presented in Fig. 8, the bared silica exhibited excellent resistance to nonspecific phosphoprotein adsorption due to its hydrophilic nature. In comparison to AGNP, a small amount of adsorbed phosphoproteins was found for SiO₂-NH₂ and SiO₂-CHO,

which is a result of electrostatic interactions between the negative-charge-carrying phosphoprotein molecules and the positive-charge-carrying nanoparticles. Although the bared silica nanoparticles had a zeta potential of -29.1 mV (Table 2), the adsorption capacity of phosphoproteins was very low and the main reason is that the nonspecific adsorption was reduced by the high ionic strength. Therefore, the high phosphoprotein adsorption capacity is attributed to the specific affinity between aluminium hydroxide and phosphoprotein molecules.

At the same time, commercial α -Al₂O₃ nanoparticles were used as a control in this experiment. As shown in Fig. 8, the binding capacities of AGNP for phosphoproteins were 10-fold higher than that of α -Al₂O₃.

Table 2 Zeta potential of nanoparticles in different pH (mV)

	6	7	8
SiO ₂	-29.1	-31.7	-36.7
SiO ₂ -CHO	30.2	23.5	9.45
AGNP	27.7	27.1	5.78

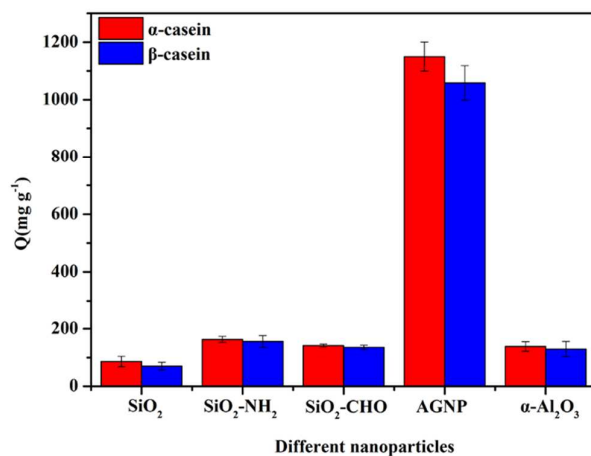


Fig. 8 Phosphoproteins adsorption capacity on SiO₂, SiO₂-NH₂, SiO₂-CHO, AGNP and α -Al₂O₃. Error bars represent the standard deviations, $n=3$.

Selective capture of phosphoproteins from standard protein mixtures

The selective capture of phosphoprotein from standard protein mixtures were tested by UV-Vis spectrophotometer. The result was showed in Fig. 9. Here β -casein was chosen as phosphoprotein, and its concentration was kept constant at 0.5 mg mL⁻¹. BHB was chosen as nonphosphoprotein. A series of protein mixtures were prepared in different mass ratio (nonphosphoprotein/phosphoprotein) at 1:1, 1:10 and 1:20 to simulate complex samples. From Fig. 9, we can observe that the amount of captured phosphoprotein increased with the mass of nonphosphoprotein in the mixture decreased and reached a maximum at 1:20 ($Q_{\beta\text{-casein}}=624$ mg mL⁻¹). In this case there was little change in the nonspecific adsorption. It was indicating that AGNP had an excellent ability in selective capture of phosphoprotein from standard protein mixtures.

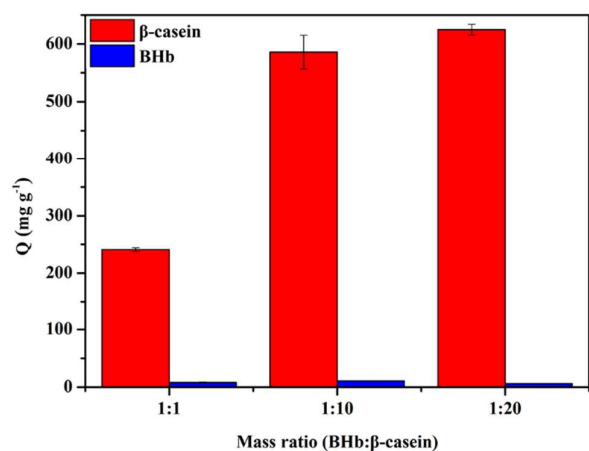


Fig. 9 The study of BHB/β-casein proteins mixtures in different mass ratios by UV-Vis spectrophotometer. Error bars represent the standard deviations, n=3.

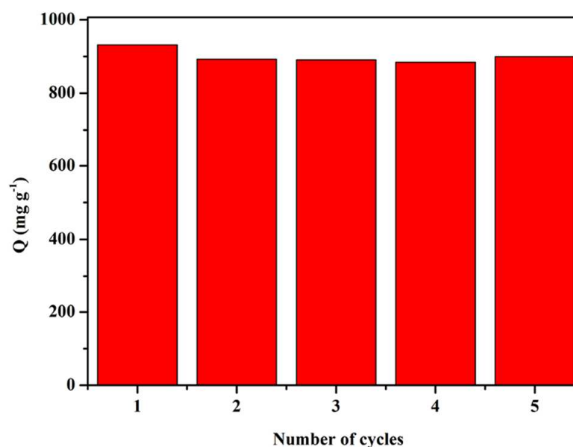


Fig. 11 Recycling experiment in the separation of phosphoprotein.

To further investigate the specificity of AGNP for the separation of phosphoproteins, a protein mixture of Lyz, BSA, α-casein was adopted. Fig. 10a shows the HPLC chromatogram of the initial protein mixture. After treatment with AGNP, 98% α-casein was removed from the mixture with minor loss of Lyz and BSA (8.7% and 6.3%, respectively) (Fig. 10b). The captured α-casein on AGNP can be eluted with 10% ammonium hydroxide, and 85.44% α-casein was recovered in the eluent (Fig. 10c).

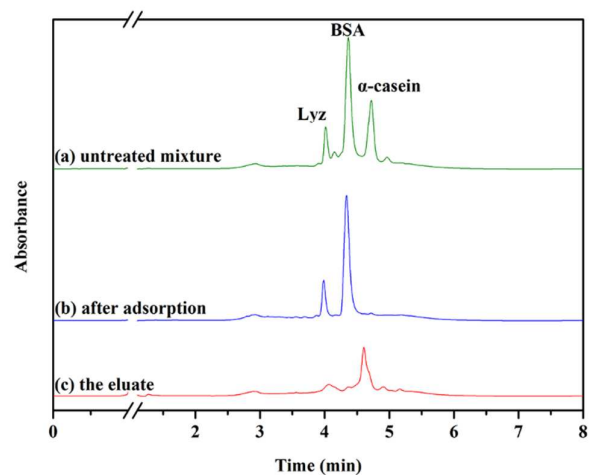


Fig. 10 HPLC chromatograms of the untreated protein mixture of α-casein, BSA and Lyz with a mass ratio of 8:14:1 (a), the supernatant treated with AGNP (b), and the eluate from AGNP-protein conjugates (c).

Reusability

The reusability of AGNP was also tested. As shown in Fig. 11, there is about 4.2% loss observed for the binding capacity of AGNP toward α-casein after adsorption-regeneration cycle was repeated five times. The result indicates that AGNP has an excellent durability and reusability.

Application

As shown in Fig. 12a, the separation of BSA/β-casein mixtures without treatment (Lane 2) revealed several major bands, which were mainly attributed to BSA and β-casein proteins. Lane 3 showed the supernatant after treatment with AGNP, in which less than 6% β-casein (quantification of the protein bands was performed with Quantity One software (Bio-Rad)) was left. Namely, nearly 94% β-casein was captured by AGNP, but the other proteins including BSA (92% remained) were almost reserved. Moreover, only the band of β-casein reappeared in the eluent of AGNP-proteins conjugates (Lane 4), showing the high selectivity of AGNP toward phosphoprotein. The recovery of β-casein was about 52% and the lose mass of β-casein is mainly attributed to the short time for elution and the few washing times.

The drinking milk was used to further probe the selectivity of AGNP in separation and enrichment of phosphoproteins from real complex samples, due to the drinking milk contains abundant proteins including phosphoproteins such as α-casein and β-casein. As described in the experimental section, drinking milk was diluted 10-fold and then incubated with AGNP. It is reasonable to expect that AGNP will show high adsorption affinity for casein.

As presented in Fig. 12b, the whole milk without treatment (Lane 2) revealed many bands corresponding to casein fraction from 19 to 35 KDa (including α-casein, β-casein and κ-casein) and whey fraction from 14 to 18 KDa (including α-lactoglobulin and β-lactoglobulin), which are the main proteins found within that range.^{30, 31} After treatment with AGNP, the supernatant (Lane 3) shows a lighter casein band, whereas the eluate (Lane 4) only presents the phosphoproteins casein band, suggesting that the phosphoprotein can be high selectively capture from the real sample. The result is similar with the reported literature.²²

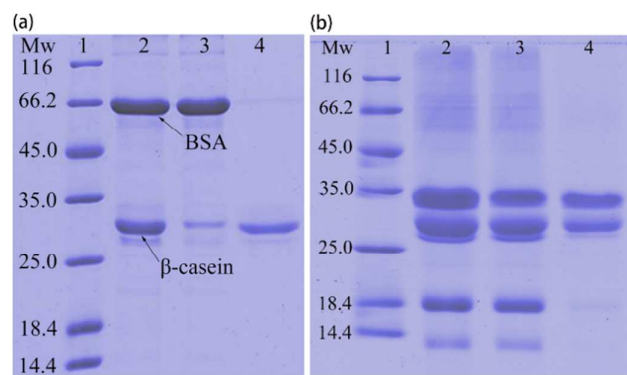


Fig. 12 (a) SDS-PAGE of BSA/ β -casein treated with AGNP: Lane 1: molecular weight marker, Lane 2: the initial protein mixtures, Lane 3: the supernatant after treatment, Lane 4: the eluate. (b) SDS-PAGE of 10-fold diluted drinking milk treated with AGNP: Lane 1: molecular weight marker, Lane 2: the initial 10-fold dilution of drinking milk, Lane 3: the supernatant after treatment, Lane 4: the eluate.

Conclusions

In summary, a facile method was developed to prepare aluminium hydroxide functionalized silica nanoparticles through a step-by-step process. The obtained AGNP offered a high surface and thus excellent binding capacity for phosphoproteins could be achieved. The adsorption studies showed that AGNP had high selective recognition. Furthermore, AGNP showed excellent recyclability. In addition, the successful application in the selective capture of casein from drinking milk suggests that the purposed method could be expected to be an alternative solution for phosphoproteomics.

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Graphical abstract

Aluminium hydroxide functionalized silica nanoparticles was synthesized by a facile approach and successfully applied for specific capture of phosphoproteins from complex sample.

