

A super hydrophilic silsesquioxane-based composite for highly selective adsorption of glycoproteins

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Abstract The authors have prepared a super-hydrophilic polymer consisting of a poly-polyhedral oligomeric silsesquioxane (POSS)-formaldehyde (PPF) composite. The polymerization process does not require a catalyst and results in a material with excellent hydrophilic properties and abundant functional groups. The PPF composite, even if not chemically modified, can selectively bind glycoproteins due to strong hydrophilic interactions. It is shown that glycoproteins can be selectively captured by the composite that has a binding capacity as large as 542 mg g⁻¹ for the model protein ovalbumin. The PPF was applied to the selective capture and isolation of ovalbumin from complex biological samples.

Keywords Poly-polyhedral oligomeric silsesquioxane-formaldehyde · Protein capture · Hydrophilic interaction · Ovalbumin · Proteomics

Introduction

Protein glycosylation plays a biologically significant role in the diagnosis and therapeutic monitoring of cancer due to its close relationship with the initiation and progression of tumors

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[1–6]. As glycoproteins usually coexist with other components in real samples, their highly specific capture/isolation from complex biological matrix becomes the prerequisite for glycoproteome analysis. In this respect, a lot of approaches have been developed for the selective enrichment of glycoproteins based on covalent interactions, e.g., hydrazine [7, 8] and boronic chemistry [9, 10], and glycan-specific recognition, e.g., lectin-affinity [11, 12]. However, these approaches frequently run into problems in practical applications, e.g. complex per-iodate oxidation process and poor efficiency in O-glycoproteins release for hydrazine chemistry [13], potential degradation of some unstable glycoproteins for boronic chemistry [10, 14] and complex immobilization process [11] and biased recognition behavior [15] for lectin affinity. In recent years, hydrophilic interaction chromatography (HILIC) is gaining increasing attention due to its broad glycan specificity, favorable reproducibility and enrichment efficacy [16–18].

In the last decades, various adsorbing materials have been designed for glycoprotein isolation based on their surface hydrophilic functional groups, e.g., amide, diol, amine, amino acid, azide, imine, hydroxyl and sulfonate groups [19]. However, tedious but necessary modification procedures are generally carried out to increase the amount of functional groups and meanwhile eliminate the interference from non-glycoproteins [20].

Owing to the existence of eight reactive peripheral functional groups, polyhedral oligomeric silsesquioxane (POSS) can provide versatile linking unites for constructing advanced materials [21–24]. Hitherto, POSS-based functional polymers have shown outstanding capability in entrapping guest molecules, i.e., fluorescent dyes [25] and organic solvents [26], by size tuning and peripheral modification. As a representative POSS, octa-amino POSS (OA-POSS) has eight primary amino groups and a well-defined cubic octameric silica cage. Amino groups, existing in the form of chloride salts, can act

as efficient conjugation sites for polymerization [27] and surface modification [28], and their hydrophilic nature endows them with great affinity towards glycan groups. In addition, Si-O-Si groups in inorganic silica cage are structurally similar with that of C-O-C in PEG, which ensures efficient resistance to non-glycoproteins. Thus, OA-POSS is an ideal molecular entity to bind glycoprotein, while its high solubility in aqueous medium makes it incapable for performing solid phase extraction.

In the present study, a poly-POSS-formaldehyde polymer (PPF) is derived from OA-POSS and paraformaldehyde by a single-step polymerization process (Scheme 1). PPF shows favorable solvent-stability, beneficial to solid phase extraction of glycoproteins. Furthermore, the intrinsic hydrophilic nature of OA-POSS is well maintained after polymerization, which endows PPF favorable glycoprotein-adsorbability and non-glycoprotein-resistance. The practicability of the product in the efficient capture of glycoprotein species has been well demonstrated, and the present practice provides an efficient approach for bio-separation.

Experimental section

Materials and reagents

Paraformaldehyde, cetane trimethyl ammonium bromide (CTAB) and dimethyl sulfoxide (DMSO) are purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China, <http://www.reagent.com.cn>), and employed as received. (3-aminopropyl)triethoxysilane (APTES) is obtained from Aladdin Chemical Reagent Co. Ltd. (Shanghai, China, <http://www.aladdin-e.com>). Acetone, methanol, acetic acid, H_3PO_4 , boric acid, and NaCl are supplied by Tianjin Damao Chemical Reagent Factory (Tianjin, China, <http://dmchem.foodqs.cn>) and at least of analytical reagent grade.

Ovalbumin (Ova, A5503, 98%, pI 4.7), immunoglobulin G from human serum (IgG, 14,506, 95%, pI 8.0), lactoferrin from bovine milk (bLf, L9507, 85%, pI 8.2), conalbumin (ConA, C7786, 98%, pI 6.8), myoglobin from equine heart (Mb,

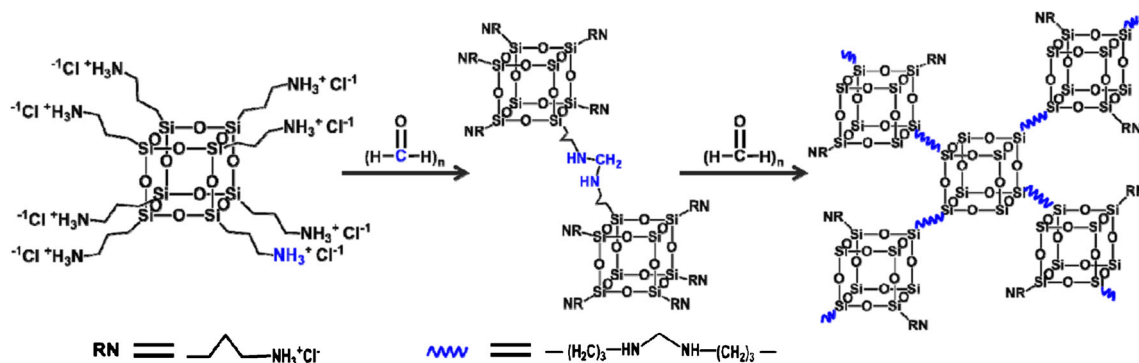
M1882, 90%, pI 7.0), cytochrome c (cyt-c, 30,398, 95%, pI 9.8), α -lactalbumin from bovine milk (α -LA, M1882, 90%, pI 4.2–4.5), lysozyme from chicken egg white (Lys, L6876, 90%, pI 11.0) and bovine serum albumin (BSA, A 3311, 98%, pI 4.9) are purchased from Sigma-Aldrich (St. Louis, USA, <http://www.sigmaaldrich.com>). γ -globulin from bovine milk (fraction II, γ -Glo, G0034, pI 8.2) is obtained from TCI (Tokyo Kasei Kogyo Co. Ltd., Japan, <http://www.teichemicals.com>). These proteins are used without further purification. The protein molecular weight marker (broad, 3452, Dalian, China, Takara Biotechnology Company, <http://www.takara.com.cn>) is a mixture of nine purified proteins (Mr in kDa: myosin, 200; β -galactosidase, 116; phosphorylase B, 97.2; serum albumin, 66.4; ovalbumin, 44.3; carbonic anhydrase, 29; trypsin inhibitor, 20.1; lysozyme, 14.3; aprotinin, 6.5).

Preparation of Octa-amino POSS

Octa-amino POSS is prepared by following a previous procedure [29]. Generally, 20 mL of APTES and 160 mL of methanol are mixed in a 500-mL beaker and stirred homogeneously. After slow addition of 27 mL of hydrochloric acid (36.5%, m/v), the mixture is continuously stirred at room temperature for 7 days. The white precipitate is collected via centrifugation and washed with methanol (10 \times) to remove any residual HCl and by-products. The product is then transferred into a vacuum oven and dried at 75 °C for overnight.

Preparation of poly-POSS-formaldehyde

528 mg of OA-POSS (0.6 mmol), 162 mg of paraformaldehyde (5.4 mmol) and 16 mL of anhydrous DMSO are mixed in a 50-mL round-bottom flask. After degassing, the mixture is heated at 180 °C for 24 h under nitrogen atmosphere. A brown precipitate is collected by centrifugation and washed with DMSO (3 \times), acetone (5 \times), methanol (5 \times) and water (3 \times) sequentially to remove the unreacted OA-POSS and paraformaldehyde. The product, shortly termed as PPF-180, is dried at 70 °C under vacuum for overnight.



Scheme 1 The schematic illustration for the preparation of poly-polyhedral oligomeric silsesquioxane (POSS)-formaldehyde (PPF)

Characterization of PPF-180

The morphology of PPF-180 is observed by scanning electron microscopy (SEM, LEO, Germany). The specific surface area of PPF-180 is measured at 77 K by a Micromeritics Tristar 3000 analyzer (USA) via nitrogen adsorption-desorption experiment. Fourier transform infrared (FT-IR) spectra of the products in KBr pellets are measured by a Nicolet 6700 spectrometer (Thermo Electron, USA) from 400 to 4000 cm^{-1} . The surface charge properties of PPF materials are collected by a ZEN3600 Nano Zetasizer (Malvern, UK). Solid-state ^{13}C NMR spectra of PPF-180 is recorded on a Bruker AVIII HD 400 MHz spectrometer (Bruker, Switzerland) to acquire its atomic-level structure. X-ray photoelectron spectroscopy (XPS) scanning curve for PPF-180 is recorded on an ESCALAB 250 surface analysis system (Thermo Electron, England). The thermogravimetric analysis (TGA) is carried out on a TGA/DSC 1 STAR^o System (Mettler-Toledo, Switzerland) from 30 to 800 $^{\circ}\text{C}$ with a heating rate of 10 $^{\circ}\text{C min}^{-1}$ under nitrogen atmosphere. The water contact angles of PPF materials are measured by a JC200D1 (Biolin Scientific, Sweden).

Protein capture and release

To evaluate the capability of PPF-180 in proteins adsorption, five glycoproteins (Ova, IgG, γ -Glo, bLf and ConA) containing different glycan structures, and five non-glycoproteins (Mb, cyt-c, α -LA, Lys and BSA) are selected as the model proteins. The capture behaviors of PPF-180 towards these protein species are investigated in 0.04 mol L^{-1} Britton-Robinson (B-R) buffer at various pH values.

Typically, 2 mg of PPF-180 is added into 1 mL of protein solution (150 $\mu\text{g mL}^{-1}$), then the mixture is shaken vigorously in a vortex vibrator for 40 min to facilitate the protein capture process. Thereafter, the supernatant is collected by centrifugation at 10000 rpm (6708 g) for 8 min for the quantification of the un-captured protein by measuring their characteristic absorption at 280 nm. PPF-180 is washed with 1 mL of deionized water to remove the loosely adsorbed proteins onto its surface, followed by mixing it with 1 mL CTAB solution (0.1 wt%) and the mixture is then oscillated to release the captured proteins. The recovery is evaluated by measuring the protein concentration in the supernatant after centrifugation at 10000 rpm for 8 min.

Results and discussion

Preparation and characterization of PPF

The OA-POSS is prepared via a hydrolytic condensation process with APTES and hydrochloric acid in methanol at room temperature. The condensation reaction between OA-POSS

and paraformaldehyde is simply carried out by refluxing their mixture under nitrogen atmosphere. After removal of the by-products, a brown solid product is obtained.

SEM image indicates that PPF-180 consists of submicron spherical particles (Fig. S1). BET analysis results indicated a specific surface area of 7.6457 $\text{m}^2 \text{g}^{-1}$ and a pore volume of 0.016045 $\text{cm}^3 \text{g}^{-1}$ for PPF-180. FT-IR spectra demonstrate that there are abundant hydrophilic groups such as amino, amide, imide and aminal groups existing on surface of PPF-180 (Fig. S2), in accordance with TGA results (Fig. S3).

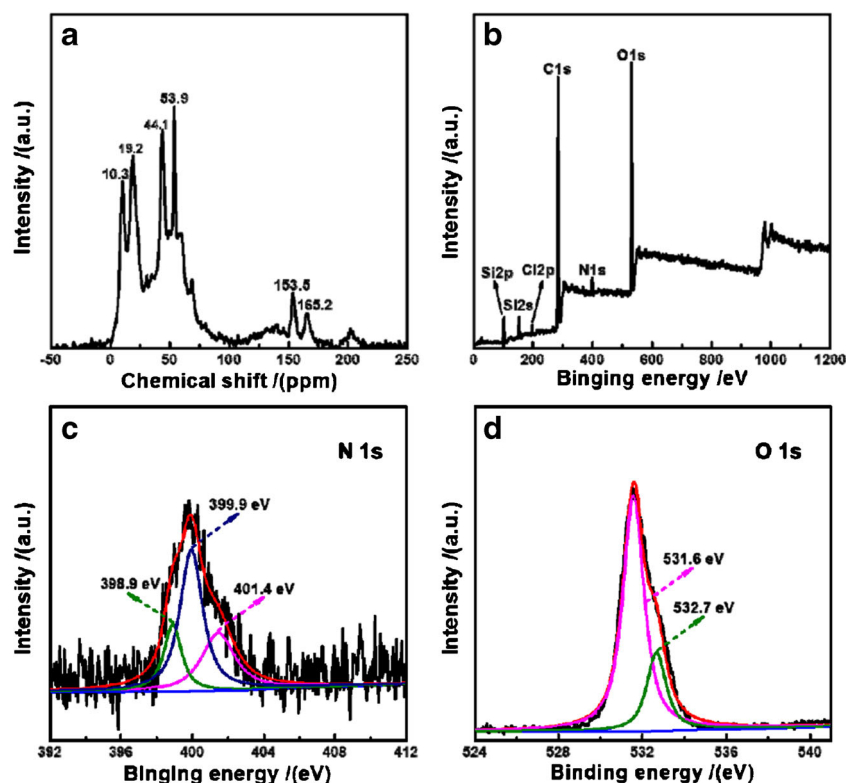
Normally, the extensive reaction at a higher temperature results in high degree of cross-linking and low solubility of the product. In this case, PPF-180 exhibits excellent solvent-stability resulting from its densely cross-linked polymer structure. This not only facilitates SPE operation, but also reduces the surface charge of the adsorbent (seen in Fig. S4), which is favorable for suppressing non-specific adsorption associated with electrostatic interaction. Thereof, the product prepared at 180 $^{\circ}\text{C}$, i.e., PPF-180 (with a zeta potential lower than 10 eV), is adopted.

In the ^{13}C NMR spectra of PPF-180 (Fig. 1a), the resonance at 165.2 and 153.2 ppm are ascribed to the carbonyl group connected to secondary amine and the tertiary carbon atoms. The strong resonance at 53.9 ppm is associated to the carbon atoms involved in aminal structure [30], indicating that the POSS unites are highly interconnected. The peaks at 44.1, 19.2 and 10.3 ppm are assigned to the sp^3 carbons of aminopropyl groups connected to POSS unites [31]. The information provided herein well confirm the existence of abundant surface hydrophilic groups and the occurrence of amidation and Schiff base reaction, in accordance with FT-IR and TGA results.

XPS spectrum of PPF-180 in Fig. 1b illustrates the presence of O 1s, N 1s, C 1s, Cl 2p, Si 2s and Si 2p peaks at 531, 400, 284, 197, 152 and 101 eV, respectively, indicating that PPF-180 is composed of C, O, Si, N and a small amount of Cl. In the high resolution N 1s spectrum (Fig. 1c), besides the C-NH_3^+ bonding from the original OA-POSS at 401.4 eV, there are two peaks at 398.9 eV and 399.9 eV, attributed to C = N and C-N-C, respectively. The O 1s spectrum can be deconvoluted into two peaks centered at 532.7 eV and 531.6 eV (Fig. 1d), which are assigned to Si-O and N-C = O/H-C = O, respectively.

The abundant functional groups on PPF-180 endows its excellent hydrophilicity. The water drops contact the PPF-180 membrane and immediately spread and flattened on the surface of PPF-180, deriving a contact angle of 0° , as clearly illustrated by Fig. S5a. On the other hand, the water contact angles for the oxidized form of PPF-180 (Oxi-PPF-180, by oxidation with KMnO_4), and the reduced form of PPF-180 (Re-PPF-180, by reduction with NaBH_4), are derived to be 26° and 46° , as given in Fig. S5b and S5c, respectively. This clearly indicates a reduction of the hydrophilicity.

Fig. 1 **a** ^{13}C NMR traces of PPF-180. **b** XPS spectra of PPF-180. **c** High resolution N 1 s XPS spectra of PPF-180. **d** High resolution O 1 s XPS spectra of PPF-180



Furthermore, FT-IR analysis results for Re-PPF-180 and Oxi-PPF-180 (Fig. S6) show an obvious increase of alkyl vibration absorption at 2950 cm^{-1} and the appearance of vibration absorption of nitril group at 1590 cm^{-1} . These observations demonstrate that both the oxidation and reduction processes produce hydrophobic groups and thus reduces the hydrophilicity of the final product.

Protein capture/adsorption behavior

To further ascertain the potential of PPF-180 for the selective adsorption of glycoproteins, its capturing behavior towards various proteins are carefully investigated at various pH values, as shown in Fig. 2a. It is seen that PPF-180 exhibits obvious stronger affinity towards glycoproteins than non-glycoproteins. Considering the existence of abundant hydroxyl groups in glycoproteins, the amount of glycan groups might be of great concern in protein capture. Ova, as a typical glycoprotein, has a solvent exposed sugar moiety consisting of 4–6 mannose residues and 2–4 N-acetyl-b-D-glucosamine residues [32]. The inherent exposure of glycan groups in Ova framework facilitates their contact with the hydrophilic groups on the surface of PPF-180, resulting in a high capturing efficiency. For IgG, it contains 30 different bi-antennary glycan structures [33] while its sugar domain is buried within the hydrophobic core between the two constant fragments [34], hindering its capture by PPF-180. γ -Glo has a similar structure framework with that of IgG, and thus it exhibits very

similar capture behavior. bLf has three effective glycosylation sites at Asn 368, Asn476 and Asn545, with one N-acetylglucosamine, one trisaccharide comprising two NAG residues and a b-1, 4-mannose (MAN) residue, and a mannose-rich hexasaccharide. Nevertheless, the inner core of this glycan chain at Asn545 is sandwiched between two domains of C-lobe [35], giving rise to a relatively low capturing efficiency. For ConA, its carbohydrate moiety consisting of three mannose and six N-acetylglucosamine residues [36] is buried under the protein surface [37], thus weakening its affinity toward PPF-180. It should be addressed that although the buried glycan groups are not accessible to the surface hydrophilic groups of PPF-180, the hydrophobic cores linked to sugar domains might be exposed as the pH value close to the isoelectric point (pI) of a specific protein. This can facilitate the contact between the glycan structure and PPF-180, resulting in higher capture efficiencies for glycoproteins than those for non-glycoproteins, as presented in Fig. 2a.

The experiments have demonstrated that the selectivity of PPF-180 for glycoproteins adsorption, while direct evidences for elucidating the contribution of hydrophilic groups are still necessary. To validate its contribution, the capture of glycoproteins is conducted by using Oxi-PPF-180 and Re-PPF-180 under the same conditions as that for PPF-180. In Fig. 2b, obvious deterioration on the capture efficiency of glycoproteins are observed, clearly demonstrating the contribution of hydrophilic interaction for the capture of glycoproteins. It is noticed that even though the hydrophilicity of PPF-180 is

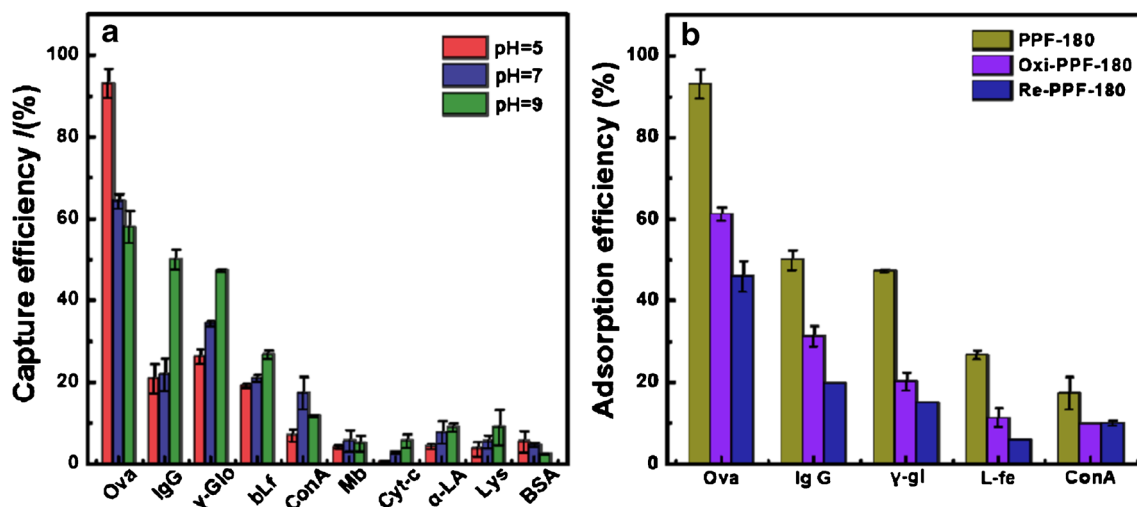


Fig. 2 a The capture efficiencies of glycoproteins (Ova, IgG, γ-Glo, bLf and ConA) and non-glycoproteins (Mb, cyt-c, α-LA, Lys and BSA) by PPF-180 at pH 5, 7, 9. b The capture efficiencies of glycoproteins (Ova, Ig G, γ-glu, L-fe and ConA) by PPF-180, Re-PPF (reduced PPF) and

Oxi-PPF (oxidized PPF) at their own optimal pH values. Concentration/volume of protein solution: 150 mg L⁻¹/1.0 mL; amount of the adsorbing material: 2.0 mg; adsorption time: 40 min

reduced by its oxidation or reduction, the capture efficiencies for glycoproteins by Oxi-PPF-180 and Re-PPF-180 are still higher than those for non-glycoproteins. This is due to the fact that some residual surface functional groups, e.g., amide and amine groups, facilitate their adsorption of glycoproteins, demonstrating the existence of abundant hydrophilic groups on PPF surface.

The effect of sample pH value on the capture efficiency is optimized. The experimental data are given in the Electronic Supplementary Material as Fig S7. A sample pH value of 5.0 is found to give the best capture efficiency. The adsorption efficiency of PPF-180 for Ova can reach 93.1%. This is ~25 times higher than that for Lys (3.5%). The exposure of glycan groups in Ova under neutral circumstance facilitates the hydrophilic interaction between protein and PPF-180, resulting in the highly selective capture of Ova.

The hydrophobic micro-environment is essential for the capture of glycoproteins by PPF-180. It is expected that the captured glycoprotein is released when there is competitive reagent. It is known that in the presence of CTAB the formed

micelle system provides a benign micro-environment for the protection of protein via hydrophobic interaction [38]. In the present study, 1 mL of 0.1 wt% CTAB solution is adopted for stripping the captured Ova and a recovery efficiency of 85% is readily achieved.

The conformation changes of proteins are investigated by use of circular dichroism (CD) spectra. As illustrated in Fig. 3a, virtually no difference is identified between the CD spectrum of the recovered Ova from PPF-180 and that of the standard Ova solution in 0.1% CTAB. This well indicated that the capture/release process poses no effect on the conformation of the protein. In addition, the reuseability of PPF-180 is evaluated by repetitive capture/release of Ova. The results in Fig. 3b demonstrated no deterioration on the capture efficiency of Ova after 5 repetitive capture/release runs. This obviously indicated that PPF-180 is suitable for repeated capture and release of proteins.

The dynamic adsorption behaviors of PPF-180, Oxi-PPF-180 and Re-PPF-180 toward Ova is investigated at room temperature within the initial concentration range of 50–900 μg

Fig. 3 a CD spectra of Ova standard solution and that recovered after the capture/stripping process by use of PPF-180 in 0.1 wt% CTAB. b The capture efficiencies of Ova by PPF-180 for 5 repeated capture-release runs

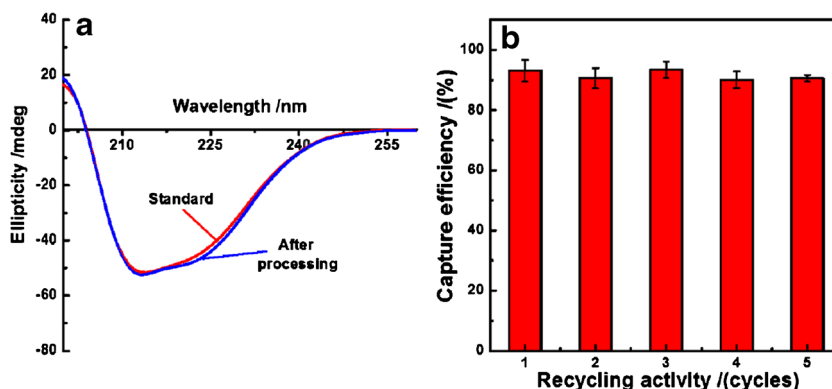


Table 1 Comparisons on performances of reported methods for the capture of glycoproteins

Materials	Driving force	Capturing capacity (mg g ⁻¹)	Ref.
Cobalt mono-substituted silicotungstic acid doping aniline (SiW ₁₁ Co/PANI)	Hydrogen bonding	200.0	[39]
Polymeric ionic liquid@SiO ₂ composite	Electrostatic attraction	333.3	[40]
Concanavalin A functionalized Fe ₃ O ₄ NPs	Lectin affinity	72.4	[11]
Benzoboroxole functionalized Fe ₃ O ₄ NPs	Boronic chemistry	93.9	[41]
Commercial hydrophilic interaction chromatography sorbent	Hydrophilic interaction	220.0	[42]
PPF-180	Hydrophilic interaction	541.8	This work

mL⁻¹ in B-R buffer at pH 5. The results (seen in Fig. S8) illustrated that the capture of Ova on PPF-180 fits Langmuir adsorption model and a maximum capture capacity for Ova is deduced to be 541.8 mg g⁻¹. For Oxi-PPF-180 and Re-PPF-180, their capture capacities of Ova are obviously decreased to 280.5 and 198.6 mg g⁻¹, respectively, demonstrating the superiority of the super-hydrophilic PPF-180 in the capture of glycoprotein. To further evaluate the adsorption performance of PPF-180, Table 1 summarizes various recently reported nanomaterial-based methods for glycoprotein adsorption. Obviously, the sorption capability of PPF-180 towards glycoprotein is superior to other adsorbents due to its abundant functional groups and super-hydrophilicity.

Selective isolation of ovalbumin from egg white

A protein mixture of Ova, IgG, α -LA and Mb is employed to testify the practical usefulness of PPF-180 for glycoproteins capture in the presence of non-glycoproteins. The supernatant

before and after treatment by 2 mg of PPF-180 and the eluent are collected for SDS-PAGE assay. Figure 4a shows that at pH 5, after treated by PPF-180 the bands for Ova and IgG are disappeared or diminished in the supernatant, while they are clearly observable in the eluent, demonstrating the selectivity of PPF-180 towards glycoproteins. On the other hand, when operating at pH 9, only Ova is retained by PPF while IgG is found in the eluent. These observations clearly demonstrate the high selectivity of PPF-180 towards glycoprotein, while it further indicates that certain extent of discrimination between glycoproteins is also possible by regulating pH value of the adsorption medium within a certain range.

The practical application of PPF-180 in the capture of glycoproteins from complex biological sample matrix is demonstrated by the selective adsorption and isolation of Ova from fresh egg white. The egg white sample is first 600-fold diluted by 0.04 mol L⁻¹ BR buffer (at pH 5), 5 mg of PPF-180 is then added into 1 mL of the egg white diluent for performing protein capture and their subsequent release/recovery process.

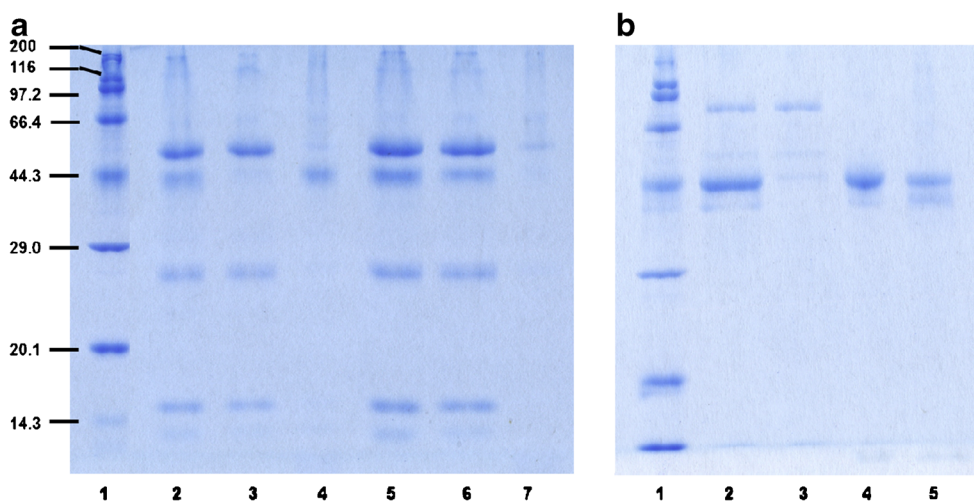


Fig. 4 **a** SDS-PAGE assay results for a protein mixture of 200 µg mL⁻¹ Ova, 100 µg mL⁻¹ IgG, Mb and α -LA. Lane 1: Marker (kDa); Lane 2: protein mixture before treatment with PPF-180 at pH 5; Lane 3: protein mixture after treatment with PPF-180 at pH 5; Lane 4: recovered protein solution after treatment with PPF-180 and stripping with 0.1% CTAB; Lane 5: protein mixture before treatment with PPF-180 at pH 9; Lane 6:

protein mixture after treatment with PPF-180 at pH 9; Lane 7: recovered protein solution after treatment with PPF-180 and stripping with 0.1% CTAB. **b** SDS-PAGE assay results for egg-white. Lane 1: Marker; Lane 2: 600-fold diluted egg white; Lane 3: 600-fold diluted egg white after adsorption by PPF-180; Lane 4: Ova recovered from PPF-180 after adsorption; Lane 5: Ova standard solution of 150 µg L⁻¹

The supernatant and the recovered protein solution by stripping with 0.1 wt% CTAB are collected for SDS-PAGE assay to confirm the feasibility of PPF-180 for the selective capture of glycoprotein. Figure 4b shows clear bands for Ova (44.3 KDa) and Lys (14.3 KDa) in the original egg white (Lane 2). After treated by PPF-180, the band of Lys remains virtually unchanged while the disappearance of Ova band is observed (Lane 3), suggesting the effective capture of Ova by PPF-180 as adsorbent. As the glycan groups of ConA is deeply buried under the protein surface, the hydrophilic interaction is rather weak between ConA and PPF-180, thus the band of ConA at 77.7 KDa is remained virtually unchanged in the supernatant after treatment with PPF-180 (Lane 3). It is obvious that for the recovered solution, only a single band is observed at the same position as that of Ova standard solution. In addition, the practical usefulness of PPF-180 for Ova in quail egg white is also investigated. As shown in Fig. S9, before performing capture/release process, more than 6 bands are seen in the original quail egg white, while only the band of Ova can be observed in the recovered protein solution. These well demonstrated the selective isolation of Ova from complex sample matrixes in the presence of other concomitant proteins.

Conclusions

We have prepared a poly-POSS-formaldehyde hybrid, PPF-180, for the selective capture of glycoproteins. The simple preparation protocol endows the achievement of POSS-based adsorbent which contains abundant functional groups and exhibits favorable hydrophilicity. PPF-180 has been demonstrated to be an efficient capturing medium for the selective isolation of glycoprotein from complex biological matrix based on hydrophilic interaction between the glycan groups of glycoproteins and surface functional groups of PPF-180. In addition, rapid capture process, high capture capacity and favorable reuseability of the adsorbent are well elucidated.

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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