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Synthesis of a SiO_2/TiO_2 hybrid boronate affinity monolithic column for specific capture of glycoproteins under neutral conditions.

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A unique boronate-functionalized SiO_2/TiO_2 hybid monolithic column was synthesized by a facile approach. Although a conventional boronic acid, 4-vinylphenylboronic acid, was used as affinity ligand, the prepared monolithic column exhibited specific capacity to capture glycoproteins including antibodies in aqueous solution at neutral pH. With the incorporation of titania, the monolith was highly hydrophilic, and the procedure of affinity chromatography could be performed under aqueous organic-solvent-free conditions.

Glycoproteins play a critical role in numerous biological processes such as cellular adhesion, signal transduction, immune defense, cell differentiation and embryonic development.[1-4] As glycoproteins are frequently present in only minute quantities and can be masked by high abundance proteins in tissue extracts and physiological fluids, great efforts have been focused on the development of glycoprotein enrichment from complex biological samples. Affinity chromatography based on the carbohydrate moiety recognition has been demonstrated an efficient method for the isolation and enrichment of glycoproteins. Lectin affinity methods have been used widely for the enrichment of glycoproteins.[5,6] Lectins are proteins that can bind to particular carbohydrate moieties through noncovalent interactions. However, different types of lectins have selective affinities for different carbohydrate epitopes of the sugar groups. When only a given lectin is used for enrichment, only a subset of glycoproteins will be captured, reducing the coverage of glycoproteins.

Affinity ligands based on boronic acid derivatives have been used to capture carbohydrates, nucleosides and glycoproteins.[7-10] The principle of boronate affinity chromatography is that boronic acids can form covalent ester bonds with *cis*-diols under basic conditions and the boronic esters can be reversibly hydrolyzed under acidic conditions. This characteristic binding specificity makes it possible to become a global approach for glycoprotein enrichment. While the use of boronic acids is regarded as one of the most promising approaches for the recognition of carbohydrate derivatives in water,[11-14] it is not without limitations. The foremost disadvantage is that a high pH (above the p*K*a of the boronic acid) is generally required in order to favor the formation of boronic esters, which may lead to an increased risk of degradation of labile molecules. A conventional solution to this challenging problem is the development of boronate ligands with lowered p*K*a values that can provide high binding affinity under neutral or even mild acidic conditions. The employment of novel boronic acids such as sulfonyl substituted phenylboronic acid,[15] Wulf-type boronic acid[16] and benzoboroxoles[17,18] had shown potentials in binding carbohydrates at a lower pH. However, there has been little success in applying them to glycoprotein enrichment.

Monoliths have been developed as a new generation of stationary phases for liquid chromatography and related techniques.[19-21] Compared with conventional particulate-packed columns, monolithic columns provide some significant advantages such as rapid convective mass transport, ease in fabrication, and low cost. Boronate affinity monolithic column inherits the virtues of boronate affinity and those of monolith. Since the first example in 2006,[22] a variety of boronate affinity monolithic columns with different properties and functions have been reported and applied for selective separation of various *cis*-diol-containing analytes.[23-27] Several monolithic columns including organic polymer-based[24] or silica-based[25] showed capacities for glycoprotein enrichment, but high pH was still required for binding.

In this work, we report on the synthesis of a unique silica/titania hybrid boronate affinity monolith by using tetrabutyl orthotitanate (TBOT), tetraethoxysilane (TEOS), *N*-(*β*-aminoethyl)-γ-amino propyltriethoxysilane (AEAPTES), and γ-methacryloxypropyltri methoxysilane (γ-MAPS) as the co-precursors and a well-known boronic acid, 4-vinylphenylboronic acid (VPBA), as the functionalized monomer. The prepared monolith is more hydrophilic, and can capture glycoproteins at physiological conditions.

The general scheme for the synthesis of the monolithic column and its recognization mechanism to the *cis*-diol-containing analytes is illustrated in Fig. 1. The formation of the silica/titania hybrid boronate affinity monolith included two major reactions, the polycondensation of the hydrolyzed precursors of TEOS, TBOT, AEAPTES and γ-MAPS, and the free-radical *co*-polymerization of the polycondensated siloxane and VPBA. Owing to the self-catalysis of the basic organic silane, AEAPTES, the hydrolyzation and polycondensation occurred rapidly and simultaneously. γ-MAPS was used as the cross-linker reagent to incorporate the boron functional moiety VPBA by the vinyl groups. The polymerization conditions such as the amounts of solvent, the ratio among precursors / monomer, and the reaction temperature were optimized. The optimal molar ratio of five initiative constituents including TEOS, TBOT, AEAPTES, γ-MAPS and VPBA was acquired. The polycondensation and

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copolymerization were carried out at 40℃and 60℃, respectively, so the fabrication of this monolith was very convenient.

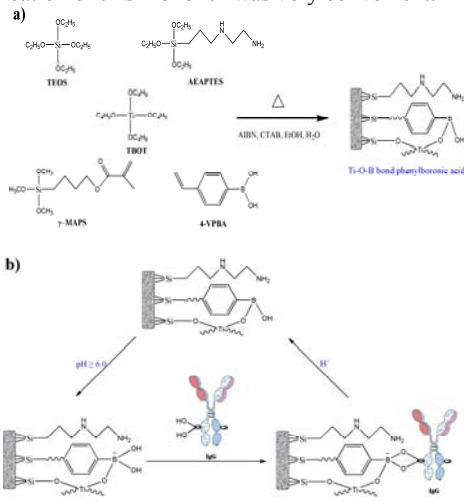

Fig. 1 (a) One-pot synthesis of silica/titania hybrid boronate affinity monolithic column, and (b) reversible capture/release of IgG.

Scanning electron microscopy (SEM) photographs of the prepared monolith revealed that the monolithic beds were anchored with the inner wall of the capillary and exhibited a well-distributed channel network (Fig. S1†). The average permeability ($K_{p,F}$) of the monolithic column was about 32.3×10^{-14} m^2 which is about 13 times higher than that reported for the column packed with particles of 5 μm ($K_{p,F}$ ~ 2.5×10^{-14} m^2).[21] The average diameter of mesopores, pore volume and specific surface area were measured by using the Brunauer-Emmett-Teller (BET) method with adsorption/desorption experiments to be 4.76 nm, 0.021cm^3 g^{-1} and 252.3 m^2 g^{-1}, respectively (Fig. S2†). The Fourier transform infrared (FTIR) spectrum for the silica/titania hybrid boronate affinity monolith is shown in Fig. S3†, and the spectrum for the silica hybrid boronate affinity monolith[28] is added as a comparison. The peaks at 1704 cm^{-1} ($\nu_{C=O}$) and 1302 cm^{-1} (ν_{C-N}) in the spectrum of silica hybrid monolith were not observed in that of silica/titania hybrid monolith. Both peaks may shift to low wave number region and be degenerated with the peaks at 1635($\nu_{C=C}$) $cm^{-1}$and 1093 cm^{-1}($\nu_{Si-O-Si}$), respectively, because of the electron-withdrawing effects of titanium (IV) ions. The peak around 955 cm^{-1} belongs to the asymmetric stretching vibration of Si-O-Ti bonds.[29] A small peak at 1390 cm^{-1} may be due to Ti-O-B vibration.[30,31] X-ray photoelectron spectroscopy (XPS) analysis indicated the monolith was made of C, N, O, Si, B and Ti (Fig. S4†). The spectrum also indicated an octahedral environment for Ti ions.[32] (Fig. S5†), and the formation of Ti-O-B bonds between boric acid and titania.[30,31](Fig. S6†). X-ray diffraction (XRD) experiments revealed that both silica hybrid and silica/titania hybrid monolithic materials were entirely amorphous.[33] The peak (Fig. S7†) shift from 2θ=21° to 2θ=23° between two materials indicated that the titanium was incorporated.[34] The elemental analysis for the silica/titania hybrid material was also performed by energy-dispersive X-ray spectroscopy (EDX) (Fig. S8†), which showed that the atom concentrations of the monolith were 8.71 % for Si and 1.00 % for Ti.

The specific affinity properties of this silica/titania hybrid monolithic column were evaluated by comparing the retention behaviors of a pair of typical structure isomers, catechol and quinol. As shown in Fig. 2, when the binding pH was set at 6.0 or higher, catechol was captured by the monolithic column while quinol had no retention. When the mobile phase was switched to 100 mM H_3PO_4, catechol was released. Other than the conventional boronate affinity columns which require a basic pH for binding, the prepared hybrid monolithic column could function at pH 6.0. A small portion of catechol could even be captured at pH as low as 5.5. The p*K*a of 4-VPBA is 8.8, but it is hard to obtain the p*K*a value of the arylboronic acid after polymerization. Based on the spectrum data and the chromatographic results, a possible mechanism was proposed as Fig. 1b. Similar to the intramolecular *o*-hydroxymethyl phenylboronic acid (benzoboroxole) strategy for carbohydrate recognition found by Hall and co-workers,[17,18] the Ti-O-B bond might be coordinated with boronate moeties to decrease the p*K*a of the boronic acid, and then could react covalently with the *cis*-diol moieties at neutral/weakly acidic aqueous environment. When the environment was changed to strong acidic condition, due to the protonation of the OH^-, the boronate esters were destroyed and the *cis*-diol-containing molecules were released from the monolith. HAc are usually employed as elution solution for boranate affinity columns. However, when 100 mM HAc was applied to elute the captured catechol from this monolithic column, a pronounced tailing was observed (Fig. S9†), which was attributable to the titania in the monolith. The unsaturated titanium (IV) ions are strong Lewis acid sites that have an affinity for the electronegative groups including hydroxyl group. And because of the stronger interaction between phosphates and titanium ions,[35] H_3PO_4 solution was expected to be appropriate for elution. Its effect was verified, and the optimal concentration was chosen by chromatographic experiment (Fig. S10†). The retention mechanism on this hybrid boronate affinity monolith was further confirmed when using deoxyadenosine and adenosine as test compounds (Fig. S11†). Adenosine is *cis*-diol containing molecule and was captured by the monolith.

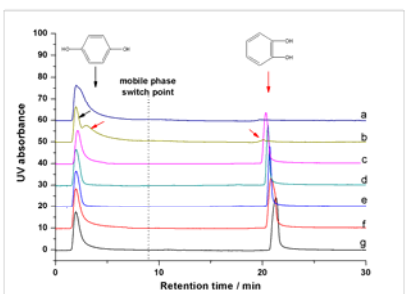

Fig. 2 Chromatographic retention of quinol and catechol on the silica/titania hybrid boronate affinity monolithic column. Mobile phase: a–g) 10 mM sodium phosphate buffer at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, respectively; the mobile phase was changed to 100 mM H_3PO_4 at 9 min. Sample: quinol and catechol were dissolved in the H_2O at a concentration of 0.2 mg mL^{-1} each.

Under physiological conditions (i.e., pH 7.0 in 10 mM PB), the functionalized monolithic column exhibited strong boronate affinity and excellent specificity not only to *cis*-diol small molecules but also to the macromolecular, glycoproteins. As shown in Fig. 3, nonglycoproteins, such as bovine serum albumin (BSA), myoglobin (Mb) and cytochrome c (Cyt C), had no chromatographic retention on the hybrid affinity monolithic column. In contrast, the glycoproteins including ovalbumin (OVA) and lactoferrin (LF) were captured selectively. Antibodies are glycoproteins and have much higher molecular weights (around 150 kD). The specific affinity of this hybrid monolith to human Immunoglobulin G (IgG) and monoclonal antibodies of mouse anti-human T lymphocytes CD25 antigen (WuTac) and mouse

anti-human T lymphocytes CD4 antigen (WuT4), was also demonstrated (Fig. 4). When IgG sample was loaded at neutral pH by four consecutive injections, and then eluted by acidic elution solution, the peak of IgG became sharper, indicating a successful enrichment of IgG. The binding capacities of the prepared monolith were measured to be 0.013 μmol mL^{-1} for IgG and 20.20 μmol mL^{-1} for catechol at pH 7.0. Antibodies are of enormous importance in basic biological research, clinical diagnosis and treatment. Purity requirements are usually exigent for antibody applications. Although protein A-based affinity chromatography is the gold standard for antibody purification, the approach also has several apparent limitations such as high cost and poor stability.[36] This silica/titania hybrid boronate affinity monolith is a promising alternative for antibody purification since it is very cheaper, highly stable and easy-to-use. While some VPBA-based affinity materials showed capacities to capture glycoproteins (typically at pH above 8.0), nonspecific adsorption of nonglycoproteins on the affinity materials frequently occurred. High concentration chaotropic salt reagents or organic solvents at different contents had to be added in the loading and elution buffers for binding and release, respectively. However, these additives increase the risk of protein denaturation and impact the further applications of the glycoproteins. Due to the strong electron withdrawing effects of titanium (IV) ions, the prepared silica/titania hybrid monolithic column was highly hydrophilic that overcome the hydrophobic interaction, and allowed for the enrichment of antibodies under aqueous organic-solvent-free conditions.

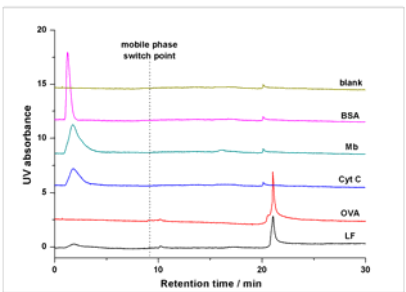

Fig. 3 Chromatographic retention of glycoproteins on the silica/titania hybrid boronate affinity monolithic column. Mobile phase: 10 mM sodium phosphate buffer (pH 7.0), the mobile phase was changed to 100 mM H_3PO_4 at 9 min. Sample: non-glycoproteins and glycoproteins were dissolved in the H_2O at a concentration of 0.5 mg mL^{-1} each.

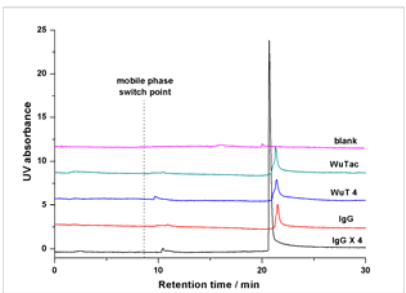

Fig. 4 Chromatographic retention of antibodies on the silica/titania hybrid boronate affinity monolithic column. Mobile phase: 10 mM sodium phosphate buffer (pH 7.0), the mobile phase was changed to 100 mM H_3PO_4 at 9 min. Sample: antibodies were dissolved in the H_2O at a concentration of 0.5 mg mL^{-1} each. IgG×4 imply continuous injections for 4 times.

In summary, a new boronate affinity monolithic column has been developed by incorporating functional boronate moieties into the silica/titania hybrid monolith. The one-pot synthesis procedure was performed under mild experimental conditions, making the preparation of the monolith convenient and simple. To our knowledge, this is the first report on a boronate-functionalized silica/titania hybrid monolithic column. Other than the strategy to synthesize and screen novel boronic acids with lower p*K*a values, a conventional boronic acid, VPBA, was used in this work. The prepared monolith was highly hydrophilic and was able to selectively capture glycoproteins including antibodies in aqueous solution at neutral pH. Since the elution was achieved under low pH conditions, future studies will focus on the development of relatively mild elution strategies and the further applications of this monolithic affinity chromatographic method.

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Table of Content

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