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High-performance liquid chromatography of amino acids, peptides and proteins

CXXX☆. Modified porous zirconia as sorbents in affinity chromatography

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ABSTRACT

The utilisation of organosilanes to introduce active chemical groups onto zirconia surfaces, suitable for the subsequent immobilisation of proteins or other biomimetic ligands, is described. Two different types of porous zirconia-based particles with nominal pore diameters of 160 and 1000 Å pore size were modified with two different affinity ligands. In the first case, methods to immobilise iminodiacetic acid–Cu(II) and its application in Cu(II) immobilised metal ion affinity chromatography (IMAC) were established. In the second series of experiments, concanavalin A was immobilised and the interaction of this lectin with the enzyme horseradish peroxidase examined. For both systems, adsorption isotherms were recorded as batch experiments. In each case, the experimental results could be fitted to langmuirean type adsorption isotherms, indicating that under the chosen conditions only one type of interaction is present, with nonspecific interactions with the support surface playing an insignificant role. These studies document the potential of surface modified zirconia particles for the immobilisation of chemical ligands or proteins for use in biospecific affinity chromatography and immobilised enzyme bioreactors.

INTRODUCTION

In recent years, research has increasingly been undertaken with metal oxides other than silica as support materials for use in the development of HPLC sorbents [1–4]. Of the various materials now available zirconia has attracted considerable interest because of its unique characteristics. Zirconia can now be manufactured in a range of particle and pore sizes and exhibits a high chemical stability from low to high pH values (*e.g.* pH 1–14). Furthermore, zirconia particles combine these favourable characteristics of chemical stability with a physical strength equal or superior to silica [5]. Therefore, microparticulate zirconia offers the benefits of an advanced support material for chromatographic sorbents. In previous studies we have documented that zirconia-based materials can be readily modified with a variety of ligands by using silane chemistry to introduce reactive groups onto the surface [6]. Additionally, pH stability tests have shown that these chemically modified adsorbents are stable up to pH 12. Another interesting feature of zirconia particles is their high density which makes them ideal for utilisation in expanded or fluidised bed systems as specific adsorbents, as immobilised enzyme bioreactors and in various other biotechnological applications. In order to document this potential, we have modified zirconia particles as bioaffinity systems to investigate the compatability of these adsorbents with proteins. In this paper, the synthesis of metal chelate- and concanavalin A-modified sorbents are described and the systems characterised by

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batch adsorption experiments. The adsorption isotherms were evaluated in terms of Langmuir-type behaviour.

MATERIALS AND METHODS

Chemicals and support material

Two types of ceramic particles based on zirconia were used in these investigations. One type of zirconia (referred to as PDZ in the text) was prepared as part of ongoing investigations on new bioceramics within the Centre for Bioprocess Technology, Monash University. PDZ zirconia was available in two particle forms with an average size of 7 μm, a pore size of 1000 Å and specific surface areas of either 1.0 or 4.2 m^2/g (measured by BET). The other type of particle was donated by the 3M company, St. Paul, MN, USA and used as a comparison to the PDZ materials. These particles had an average particle size of 15 μm, a pore size of 160 Å and a surface area of 32 m^2/g.

Iminodiacetic acid, concanavalin A (Type IV, from Jack Bean), horseradish peroxidase (E.C. 1.11.1.7, Type II) and myoglobin (Type III, from horse heart) were purchased from Sigma, St. Louis, MO, USA; 3-glycidoxipropyl-trimethoxysilane and 3-isothiocyanatopropyl-triethoxysilane were purchased from Fluka, Germany. All other chemicals were purchased from BDH, Melbourne, Australia and were of analytical grade.

Pretreatment of the zirconia particles

In a previous paper the optimum pretreatment of zirconia particles was investigated [6]. Accordingly, all particles were hydrothermal treated for 6 h at 150°C prior to chemical modification.

Modification with iminodiacetic acid

The preparation of the iminodiacetic acid (IDA) silane was based on a procedure developed by Anspach [7]. In brief, iminodiacetic acid (1 g) and NaOH (1.530 g) were dissolved in 18 ml water and the solution was cooled in an ice bath. 3-Glycidoxypropyltrimethoxysilane (1.776 g) was then added dropwise. The stirred solution was allowed to warm to room temperature and then heated at 60°C overnight.

To modify the zirconia particles, five times the amount of IDA-silane required to achieve a theoretical surface coverage of 4 $\mu mol/m^2$ was employed in 20 ml of an aqueous solution adjusted to pH 3.0 with HCl for each g of zirconia particles suspended in this solution. The suspension was then heated to 90°C for 3 h.

The chemically modified particles were then washed with 100 ml of each 0.1 *M* hydrochloric acid, water and 2-propanol. The particles were air-dried and then suspended in a solution of copper sulphate (5 g/l) to saturate the IDA-chelate groups with Cu(II) ions.

Sodium phosphate (20 m*M*, pH 7.0) was employed in the equilibration and regeneration solution resulting in interactions between the phosphate ion and Lewis acid sites on the zirconia surface [8,9]. However, the stability constant for the copper-iminodiacetic acid complex is *ca.* $4 \cdot 10^{10}$ compared to *ca.* 20 and $2 \cdot 10^3$ for the copper–dihydrogenphosphate and copper–hydrogenphosphate complexes, respectively [10]. The higher stability by 10^7 of the Cu(II)–IDA complex would suggest that a possible phosphate-copper ion layer at the zirconia surface will play an insignificant role in the adsorption behaviour with proteins. Moreover, 20 m*M* phosphate was also included in the adsorption buffer, so it is very likely that any Cu^{2+} ions which inadvertently leach from the sorbent will be complexed in the bulk solution.

Modification with Concanavalin A (Con A)

Modification of the zirconia particles with Con A involved two steps [11]. Firstly, the support material was chemically modified with 3-isothiocyanatopropyltriethoxysilane (ITCPS) to introduce reactive groups onto the zirconia surface. Con A was attached via the NCS groups using the free amino groups accessible on the protein surface.

To modify the zirconia support with the silane, the particles were dried at 180°C under vacuum. Toluene was dried over sodium metal and freshly distilled. The particles were suspended in the toluene and the ITCPS silane was added. The amount of silane used per gram zirconia was

calculated on the basis to achieve a ligand density of 8 μmol/m^2 support surface area, *e.g.* twice the amount of silane that can theoretically be immobilised based on steric considerations. A small amount of imidazole (about 1%, w/w) was added as a catalyst. The suspension was sonicated for five minutes to remove air trapped inside the pores. The mixture was then heated under reflux for 24 h and then washed with 100 ml each of toluene, 2-propanol and water.

To attach the Con A, the modified particles were suspended in 50 m*M* NaOAc buffer pH 6.5 containing 10 m*M* of each of $MnCl_2$, $MgCl_2$ and $CaCl_2$. The suspension was gently agitated by inverse shaking at room temperature for 48 h and then washed with the same buffer. The particles were not dried. To block any unreacted NCS groups, the Con A-modified particles were treated with a 1% solution of ethanolamine in the modification buffer described above at pH 7.0 overnight.

Batch adsorption experiments with metal chelate-modified zirconia

The batch experiments were carried out with the instrumental system described by Anspach *et al.* [12] with modifications [13,14]. The equipment layout of this system is shown in Fig. 1.

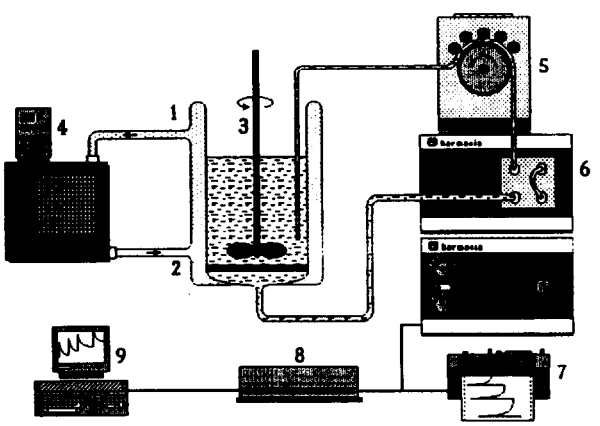

Fig. 1. Experimental setup for the recording of batch adsorption isotherms. 1 = Adsorption vessel; 2 = 1.5-μm filter; 3 = overhead stirrer; 4 = thermostat; 5 = peristaltic pump; 6 = UV detector; 7 = chart recorder; 8 = A/D converter; 9 = Atari ST-1040 computer.

In brief, the IDA-modified zirconias saturated with Cu^{2+} were suspended in 20 ml of 20 m*M* sodium phosphate buffer pH 7.0 containing 0.2 *M* NaCl. Due to the differences in the specific surface areas between the two types of zirconia particles, typically 1-g aliquots of the sorbent derived from the 3M material and 2-g aliquots of the sorbent derived from the PDZ materials were used for each of the adsorption experiments. Horse heart myoglobin was dissolved in the same buffer used to suspend the IMAC sorbents at a concentration of 1 mg/ml and added successively to the metal chelate sorbents suspended in the recycling adsorption chamber. During the experiments the temperature of the suspension was thermostated at 7°C. The rate of adsorption was monitored at 280 nm and recorded until equilibrium was reached. The equilibrium concentrations were used to calculate the adsorption isotherms which were evaluated using three different linearisation approaches, namely the double reciprocal plot, semi reciprocal plot and Scatchard plot [15–20].

Batch adsorption experiments with concanavalin A-modified zirconia

For the adsorption of horseradish peroxidase to Con A-modified zirconia at 20 m*M* sodium hydrogenphosphate–dihydrogenphosphate buffer containing 0.2 *M* NaCl, pH 6.5, was used. The buffer also contained 1 m*M* of each $CaCl_2$, $MnCl_2$ and $MgCl_2$ to maintain the biological activity of the Con A. The buffer was filtered prior to use to remove undissolved manganese or calcium phosphate precipitates. As before, either 1 g of the 3M zirconia- or 2 g of the PDZ zirconia-based sorbents were used for each experiment. The particles were suspended in 25 ml of the buffer and the suspension was thermostated at 7°C. Horseradish peroxidase was dissolved in the same buffer at a concentration of 1 mg/ml. To examine whether the binding was due to specific interaction, the adsorbate was eluted with methyl α-D-mannopyranoside and a second adsorbtion isotherm recorded. Evaluation of the experimental adsorption data utilised the same numerical approaches as in case of the metal chelate-modified supports.

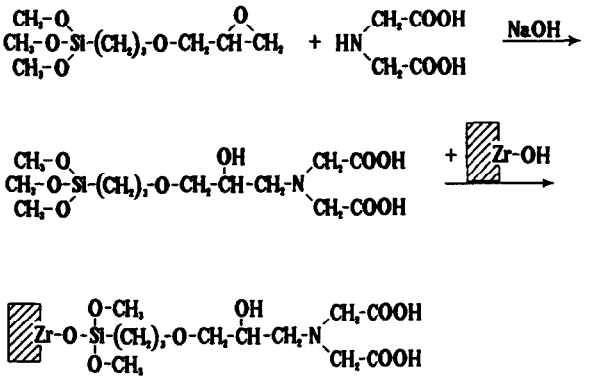

Fig. 2. Modification of zirconia with iminodiacetic acid.

RESULTS AND DISCUSSION

Porous, amorphous silica is the most commonly used of all the inorganic support materials in HPLC applications, although in recent years porous active aluminas, containing γ-alumina as the major component, have also attracted attention [21–24]. Although the surface chemistry of porous silica and γ-alumina are distinctly different in terms of the character and distribution of their Lewis acid and Lewis base sites [25], both inorganic oxides can be surface derivatised via isolated Brønsted hydroxyl groups with monofunctional reagents. With regard to the other inorganic oxides (magnesia, titania, ceria and zirconia) these oxides find very widespread applications as pigments, industrial adsorbents, dispersants and carriers, and in various (but limited) applications in TLC and GLC. These support materials have yet to find general application in HPLC. In fact, as recently as 1990, Unger stated in a detailed review [26] on adsorbents in column liquid chromatography, that no application of zirconia or seria had yet been reported on their use in high-resolution liquid chromatography.

Over the past several years, methods for the preparation of porous zirconias with defined particle size from hydrolysis of zirconyl chloride or zirconium alkoxides by thermal decomposition of zirconyl chloride [27] or by a thermal processes from zircon powder have been developed and the properties of these zirconia-based materials as high-performance chromatographic sorbents are now starting to undergo evaluation.

In this investigation we describe the facile derivatisation of porous zirconia using silane chemistry and describe some of their properties as high performance IMAC and biospecific affinity chromatographic (BAC) sorbents.

The synthetic routes to the IDA–Cu(II)–zirconia and the Con A–zirconia are shown as schemata in Figs. 2 and 3. In the case of the immobilisation of the iminodiacetic acid ligand a 5-fold excess (calculated for a maximum possible ligand density of 4 μmol/m^2 surface area) of the silylation reagent, prepared from a reaction of iminodiacetic acid and 3-glycidoxypropyltrimethoxysilane was employed with a subsequent chelation with Cu(II) ions. In the case of Con A–zirconia sorbents, a 2-fold excess of 3-isothiocyanatopropyltriethoxysilane was used to introduce reactive isothiocyanate groups onto the zirconia surface followed by the immobilisation of Con A, primarily via ε-amino groups on accessible lysine residues.

The adsorption of solutes onto the sorbent surface was evaluated in terms of langmuirean type adsorption behaviour. The Langmuir model

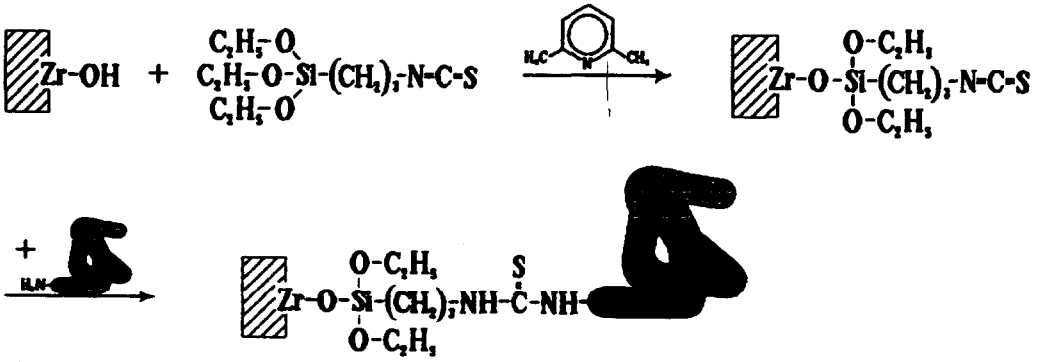

Fig. 3. Modification of zirconia with Concanavalin A.

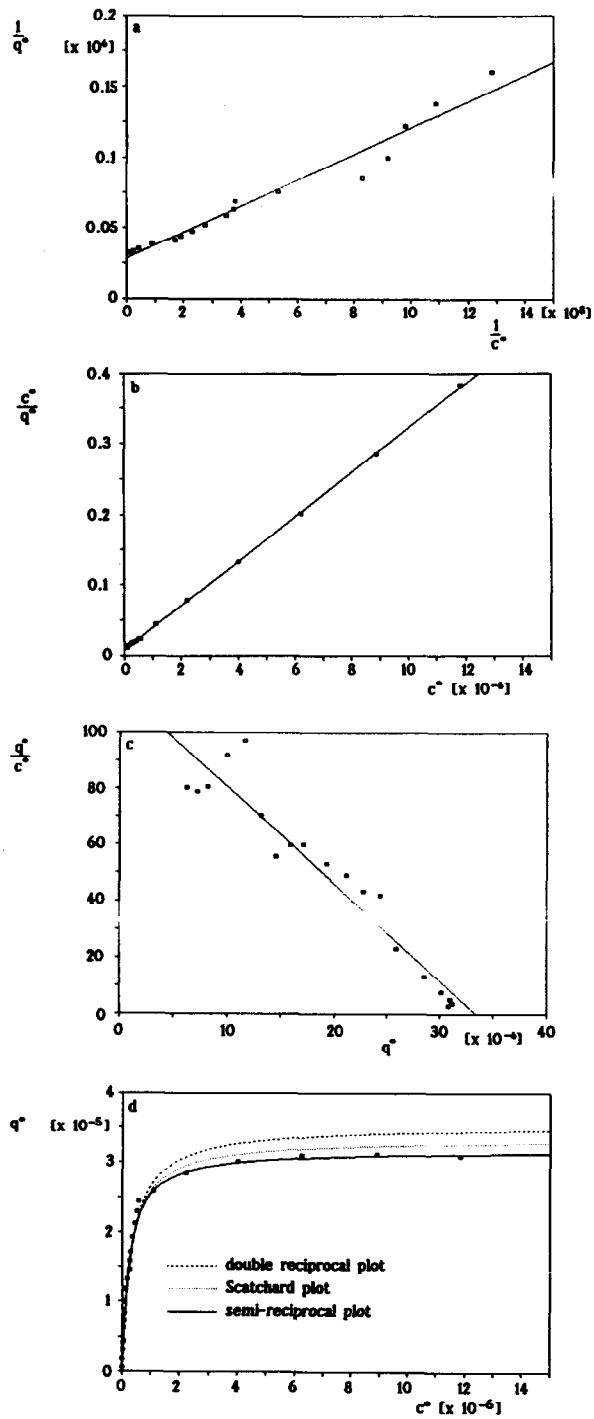

Fig. 4. Adsorption isotherm of myoglobin adsorbed onto IDA–Cu(II)-modified 3M zirconia particles, where c^* is the adsorbate concentration in solution and q^* is the amount of adsorbate on the particles. (a) Double reciprocal plot (Benesi–Hildebrandt plot), (b) semi reciprocal plot (Scott plot), (c) Scatchard plot, (d) direct plot of the isotherm.

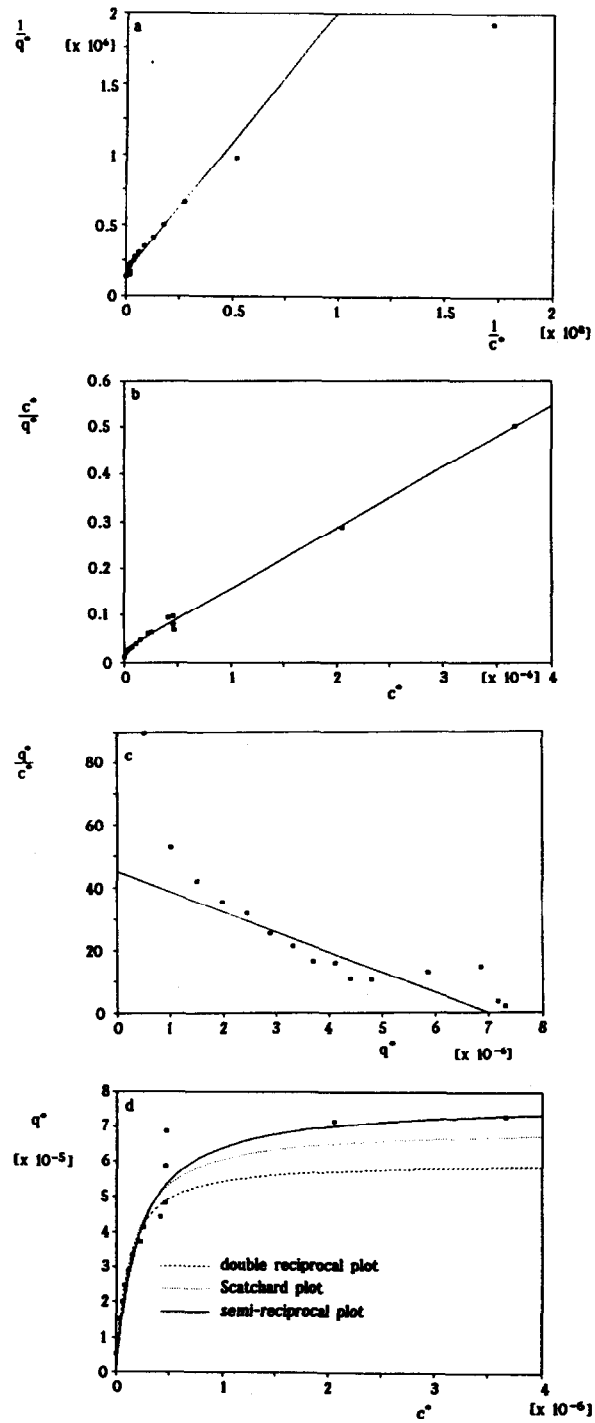

Fig. 5. Adsorption isotherm of myoglobin adsorbed onto IDA–Cu(II)-modified PDZ zirconia particles, where c^* is the adsorbate concentration in solution and q^* is the amount of adsorbate on the particles. (a) Double reciprocal plot (Benesi–Hildebrandt plot), (b) semi-reciprocal plot (Scott plot), (c) Scatchard plot, (d) direct plot of the isotherm.

makes three assumptions to simplify the adsorption behaviour: (a) All binding sites are of equal energy, (b) any binding site adsorbs only one solute molecule, and (c) a molecule adsorbed onto one binding site does not influence the adsorption of another molecule on the neighbouring binding site. Since deviations from the ideal binding behaviour are often difficult to detect, different methods of linearisation were employed, each putting emphasis on a different part of the isotherm. A combined use of these linearisation methods therefore guarantees an optimum evaluation of the adsorption behaviour.

The results of the adsorption of myoglobin with the different IDA–Cu(II)-modified zirconias are shown in Figs. 4 and 5, respectively. As evident from the three different approaches used to evaluate the adsorption isotherm data (double reciprocal, semi reciprocal and Scatchard plot) differences in the calculated values for the capacity and the association constant arise, depending on which method is employed. These differences are due to the different contributions which the experimental data make in the determination of the respective regression coefficients for the three different linearisation approaches. The semireciprocal plot exhibited the best fit of the experimental data because the adsorption values for the proteins at the higher concentrations contribute more significantly to the regression analysis than the lower concentration values. In comparison, the double reciprocal plot showed the lowest correlation for a linearised Langmuir-like adsorption because this plot emphasises more heavily the very low concentration value range where the systematic signal-to-noise ratio is much lower. Thus, in the early stages of the adsorption experiments, when only comparatively small amounts of protein were added to the system and the IDA–Cu(II)–zirconia and the Con A–zirconia were far from saturation, almost all protein in the recycling fluid was adsorbed and the free concentration of the protein was very close to the detection limit of the UV monitor. For example the detector readout after the first injection of myoglobin was 0.15 mV with a noise background of ±0.05 mV while at the same time the value for q^* was 13.45 mV.

The linear nature of both the double reciprocal and the semireciprocal plot however is indicative that the adsorption behaviour can be described in terms of langmuirean-type adsorption. The results for q_m and K_d derived from these plots are listed in Table I. The dissociation constant of the Cu(II)–myoglobin complex of the 3M material appeared to be consistently higher than the constant for the PDZ zirconia showing the influence of the restricted pore diffusion inside the 160 Å pores of the 3M zirconia.

As expected, the utilisation of a zirconia with

TABLE I

VALUES OF THE DISSOCIATION CONSTANTS (K_d) AND CAPACITY VALUES (q_m [mol/$g_{zirconia}$]) FOR MYOGLOBIN ADSORPTION ON IDA–Cu(II)-MODIFIED ZIRCONIA

	Double reciprocal plot	Semi-reciprocal plot	Scatchard plot
3M zirconia			
All data used	$q_m = 0.0736$ $K_d = 5.09 \cdot 10^{-4}$	$q_m = 6.38 \cdot 10^{-5}$ $K_d = 2.83 \cdot 10^{-7}$	$q_m = 7.16 \cdot 10^{-5}$ $K_d = 3.61 \cdot 10^{-7}$
−5 smallest concentrations	$q_m = 7.06 \cdot 10^{-5}$ $K_d = 3.27 \cdot 10^{-7}$	$q_m = 6.36 \cdot 10^{-5}$ $K_d = 2.49 \cdot 10^{-7}$	$q_m = 6.68 \cdot 10^{-5}$ $K_d = 2.89 \cdot 10^{-7}$
PDZ zirconia			
All data used	$q_m = 8.92 \cdot 10^{-6}$ $K_d = 4.65 \cdot 10^{-8}$	$q_m = 1.53 \cdot 10^{-5}$ $K_d = 1.76 \cdot 10^{-7}$	$q_m = 1.37 \cdot 10^{-5}$ $K_d = 1.16 \cdot 10^{-7}$
Data neglected as indicated in figures	$q_m = 1.21 \cdot 10^{-5}$ $K_d = 1.12 \cdot 10^{-7}$	$q_m = 1.55 \cdot 10^{-5}$ $K_d = 2.12 \cdot 10^{-7}$	$q_m = 1.40 \cdot 10^{-5}$ $K_d = 1.55 \cdot 10^{-7}$

a narrow pore size (*e.g.* the 3M zirconia) for bioaffinity adsorption of horseradish peroxidase on immobilised Con A appears to have limited practical use due to a significant reduction of the pore size by the ligand and the resulting very restricted pore diffusion of the adsorbate. This effect is particularly noticeable when the adsorption kinetics for the adsorption of myoglobin onto IDA–Cu(II)–zirconia is compared with the adsorption of horseradish peroxidase onto Con A-modified PDZ particles (Fig. 6). Investigation of the adsorption behaviour of horseradish peroxidase with Con A-modified 3M zirconia gave limited experimental results again due to the small pore size of this sorbent.

An increase in temperature from 7 to 25°C in order to significantly increase the diffusion kinetics had little effect on the shape of the isotherm. The adsorption isotherm for the PDZ zirconia modified with Con A are shown in Fig. 7 and the results for q_m and K_d are listed in Table II. The good concordance between the first experiment on the adsorption of horseradish peroxidase to Con A-modified PDZ zirconia and subsequent experiments after specific elution indicates that the binding of the horseradish peroxidase to the Con A sorbent is due to specific interactions between the carbohydrate binding site of Con A and the polysaccharide chains of the glycosylated horse radish peroxidase. Moreover, the equivalence of the capacity in these subsequent experiments indicates that the elution step completely regenerates the original capacity.

In the above experiments, phosphate-based buffers were used for adsorption and elution of the proteins. The strong interaction between

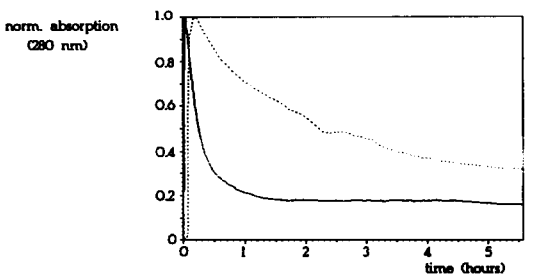

Fig. 6. Comparison of the adsorption kinetics on IDA- and Con A-modified 3M zirconia. Dotted line = Con A–peroxidase; solid line = IDA–Cu(II)–myoglobin.

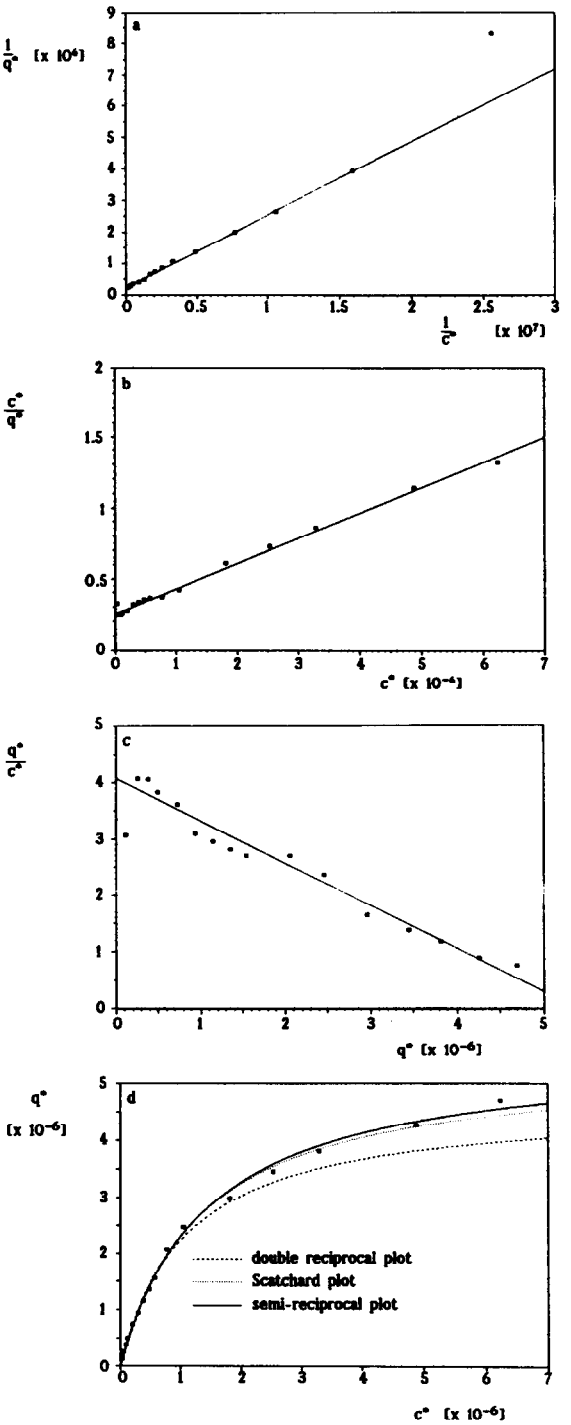

Fig. 7. Adsorption isotherm of myoglobin adsorbed onto Con A-modified PDZ zirconia particles. (a) double reciprocal plot (Benesi–Hildebrandt plot), (b) semi-reciprocal plot (Scott plot), (c) Scatchard plot, (d) direct plot of the isotherm.

TABLE II

VALUES OF THE DISSOCIATION CONSTANTS (K_d) AND CAPACITY VALUES (q_m [mol/$g_{zirconia}$]) FOR HORSERADISH PEROXIDASE ADSORPTION ON Con A-MODIFIED ZIRCONIA

	Double reciprocal plot	Semi-reciprocal plot	Scatchard plot
PDZ zirconia modified with Con A, first adsorption experiment			
All data used	$q_m = 3.52 \cdot 10^{-5}$ $K_d = 5.16 \cdot 10^{-6}$	$q_m = 1.13 \cdot 10^{-5}$ $K_d = 1.48 \cdot 10^{-6}$	$q_m = 1.11 \cdot 10^{-5}$ $K_d = 1.42 \cdot 10^{-6}$
−smallest concentration	$q_m = 9.32 \cdot 10^{-6}$ $K_d = 1.08 \cdot 10^{-6}$	$q_m = 1.12 \cdot 10^{-5}$ $K_d = 1.43 \cdot 10^{-6}$	$q_m = 1.08 \cdot 10^{-5}$ $K_d = 1.33 \cdot 10^{-6}$
Second adsorption after elution with α-methylmannose			
All data used	$q_m = 2.64 \cdot 10^{-5}$ $K_d = 6.35 \cdot 10^{-6}$	$q_m = 9.84 \cdot 10^{-6}$ $K_d = 1.97 \cdot 10^{-6}$	$q_m = 1.26 \cdot 10^{-5}$ $K_d = 2.70 \cdot 10^{-6}$

zirconia Lewis acid sites and phosphate ions was used to suppress non-specific interactions between the support surface and proteins. In particular, this interaction will suppress Lewis/Brønsted acid sites which could lead to non-specific interactions between the IDA–Cu(II)– or the Con A–zirconia surface and the test proteins. As the value of the association constant for the Con A–horse radish peroxidase complex is very similar to that obtained from bulk solution measurements [28], the conclusion can be drawn that these surface-modified zirconias interact specifically. Similar conclusions can be drawn from the myoglobin IDA–Cu(II)–zirconia interactions, where again the association constant is similar to values obtained with other IDA–Cu(II) sorbents [29].

CONCLUSIONS

The experimental results described in this paper show that the modification chemistries developed in our associated studies for various other zirconia-based sorbents [6] can also be applied to synthesise biomimetic and biospecific affinity supports. Immobilised Con A retains its biological activity as shown by the specific elution of horseradish peroxidase with methyl α-D-mannopyranoside. Both the specific and complete elution of horseradish peroxidase and the Langmuir type adsorption isotherms obtained with this system as well as the IDA–Cu(II)–myoglobin system indicate a single type of interaction. Elsewhere, the application of these new support materials with other immobilised enzymes will be described.

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