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Comparison of modified supports on the base of glycoprotein interaction studies and of adsorption investigations

Henning Rosenfeld[a,*], Jolita Aniulyte[b], Heike Helmholz[a], Jolanta Liesiene[b], Peter Thiesen[c], Bernd Niemeyer[a,c], Andreas Prange[a]

[a] *Institute for Coastal Research/Marine Bioanalytic Chemistry, GKSS Research Centre, Max-Planck-Strasse, D-21502 Geesthacht, Germany*
[b] *Department of Chemical Technology, Kaunas University of Technology, Radvilenu pl. 19, LT-50254 Kaunas, Lithuania*
[c] *Institute of Thermodynamics, Helmut-Schmidt-University/University of Federal Armed Forces Hamburg, Holstenhofweg 85, D-22043 Hamburg, Germany*

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Abstract

The features of matrices, suitable for affinity chromatography, have been extensively investigated and got subject for several reviews. But these investigations show, that there is still a lack in adsorbent characterization and a demand of comparative investigations of adsorbents, based on different materials with a range of different surface functionalities. In this work the performance of self-prepared silica and cellulose-based adsorbents were compared with commercially available polymeric supports. A model system was chosen comprising the lectins concanavalin A (ConA) and wheat germ agglutinin (WGA), which were covalently attached to the support matrices, combining the selectivity of the lectin–sugar interaction with the chemical and mechanical properties of the support that influence the efficacy of the prepared adsorbent. The verification of the different supports provides information about tayloring carbohydrate specific lectin adsorbents. The characterization outlines the main features of the different adsorbents and takes into account the properties of the pure supports. It encompasses immobilization kinetics and isotherms as well as the description of the binding capacity of the adsorbents by depicting adsorption isotherms. The separation performances were also investigated in terms of glycoprotein purification factors and recoveries. Further, detailed information about binding of GOD to immobilized ConA are obtained.

Keywords: Supports; Surface modification; Affinity separation; Selective adsorbents; Lectin; ConA; WGA; Glycoprotein purification; Silica; Cellulose

1. Introduction

Affinity adsorbents are a decisive factor for the success of affinity separation. They comprise a porous beaded matrix and covalently attached ligand molecules onto its surface. With the immobilization of the ligand molecule to the support material, the specific interaction properties of the ligand and the physico-chemical properties of the support are combined. The combination offers the possibility to generate taylored adsorbents for high specific separation processes under different conditions.

Desirable features of the basic support material are physical, chemical and biological stabilities. Examples of physical factors are high solidness, consistent particle form, sufficient permeability and low pressure resistance [1,2]. Particulary the pore structure of the solid phase should be considered carefully. It is important to adjust the pore size of the support to the dimensions of the ligand-adsorptive system, because the space occupied by a ligand is a crucial factor for the capacity of the affinity adsorbent, so that the ligand must not be restricted to the periphery of the particles. The pore size has to be large enough to allow free diffusion of proteins in and out of the bead.

In general, beneficial effects derive from a large accessible surface area with a hydrophilic character free of hydrophobic binding sites to minimize non-specific adsorption in aqueous systems. The activation method depends on the functional groups on the surface of the matrix and of the ligand. In order to immobilize proteins covalently under gentle condi-

* Corresponding author. Tel.: +49 4152 87 2831; fax: +49 4152 87 1875.
E-mail address: henning.rosenfeld@gkss.de (H. Rosenfeld).

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tions, an activation of the matrix is required. Starting with surface bound hydroxyl-, amino-, thiol-, aldehyde- or carboxylgroups, a number of activation protocols is available. Hydroxyl groups can be activated with cyanogen bromide [3], Bisepoxirane [4], organic sulphonyl chlorides [5–7], or carbodiimidazole [8]; amino groups with glutaraldehyde, carboxyl groups and with *N*-hydroxysuccinimid [9].

Protein immobilization occurs in the majority of cases via reaction of the surface-bound active groups with the lysine sidechain amino groups of the protein. Because of this reason, the number and the distribution of lysine groups in the ligand molecule is a usefull tool in affinity adsorbent engineering.

A critical point is the chemical stability of the ligand. Agressive coupling conditions or prolonged immoblilization times may render the ligand inactive and reduce the binding capacity. The influence of the nature of the ligand attachment becomes intelligible in the work of Masárová et al. They used mild immobilization conditions for the preparation of concanavalin A (ConA) adsorbents via hydrophobic interactions and received an improved activity compared to covalently immobilized ConA [10]. However, it is questionable, whether the hydrophobic link is stable in a competitive environment with other proteins. The use of spacer molecules ensures that steric limitations are kept to a minimum, which is especially important considering the size of the affinity ligands. A second benefit arising from the use of a spacer is that the ligand is kept away from the particle surface, thus its protein surface interactions can be minimized.

Desirable criteria of the affinity adsorbent are a high specificity, a good chemical stability for the procedures of the separation cycle such as adsorption, washing, desorption and regeneration of the process as well as good reproducibility and recovery for the compound to be separated, coupled with low preparation cost and high binding capacity [11]. The surface coverage with ligands on the solid-phase surface will affect working capacities up to a point where steric hindrance (particulary between macromolecular ligands and products) may diminish the performance. Investigations about the optimum ligand density of ConA adsorbents were done by Fraguas et. al. [12]. Wirth et. al. showed, that the adsorption of ConA dimers or tetramers at metal-chelate affinity supports is dependant on both the protein concentration as well as the ligand density [13]. Orientation of the ligand will also generally influence the efficiency of interaction with the products. Commonly adopted methods of solid phase coupling through surface amino groups necessarily impose a random orientation of the immobilized ligand with an impact upon the performance of any affinity adsorbent. Fig. 1 displays in dark colour the lysine moieties in the molecules that are potential attachment sites for the immobilization. It becomes obvious that the covalent attachment of the lectins onto adsobent surfaces results in different degrees of accessibility of the carbohydrate recognition domain.

Affinity chromatograms in combination with gel electrophoresis of the different collected fractions provide means to characterize affinity adsorbents, which quantify the capacity of the column under dynamic conditions. Developing affinity adsorbents, the amount of interactive ligands under dynamic separation process conditions is an important characteristic of a support, especially for high priced ligands. To evaluate this parameter, firstly immobilization isotherms and affinity interaction isotherms under equilibrium conditions and secondly chromatograms under dynamic conditions are required. Additional information of the K_d-values result from the shapes of the affinity interaction isotherms. The front of a breakthrough curve is depending on the initial and feed concentration and on the shape of the isotherm [14]. According to adsorption isotherm classification of Giles et al. [15] L1- and L2-type isotherms are correlated to breakthrough curves with a self-sharpening front and a time decreasing mass transfer

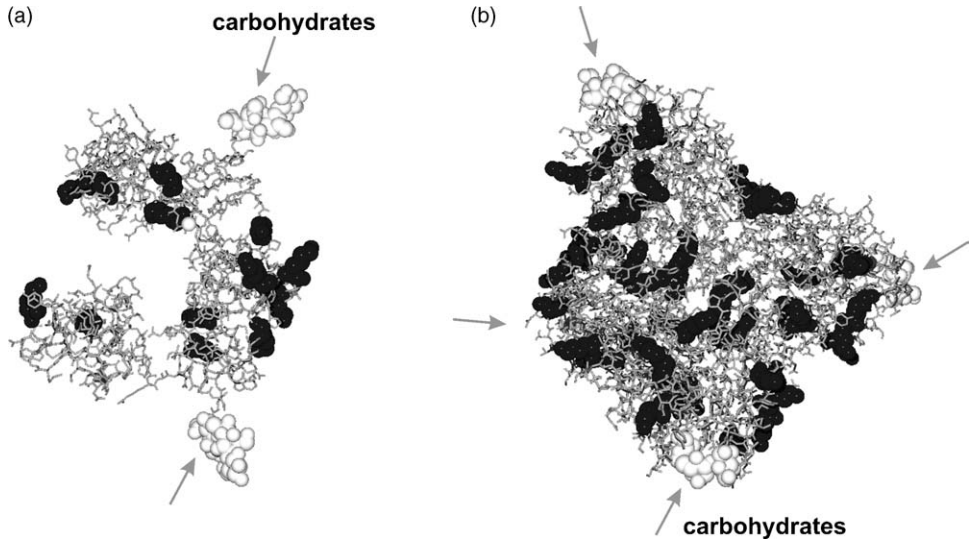

Fig. 1. Shape and lysine group distribution of the lectins: (a) wheat germ agglutinin *7wga.pdb* and (b) concanavalin A *5cons.pdb*. The lysine groups are drawn in black, the carbohydrate molecules in the carbohydrat recognion sites in gray, marked with arrows.

Table 1
Characteristics and molecular properties of the lectins concanavalin A and wheat germ agglutinin

	Concanavalin A	Wheat germ agglutinin
Abbreviation	ConA	WGA
Origin (common name)	Jack bean	Wheat germ
Latin name	*Canavalia ensiformis*	*Triticum vulgaris*
Molar mass (Da)	108,000	36,000
No.of subunits	4	2
No. of lysine-groups (monomere)	48 (12)	14 (7)
Affinity	Methyl-α-D-mannose > α-D-mannose (Man) > α-D-glucose (Glc)	*N*-acetyl-glucosamine (GlcNAc) > *N*-acetyl-neuraminic acid (NeuAc)
Inhibitor/eluting sugar	Methyl-α-mannopyranoside > Man > Glc > GlcNAc	Chitotriose > chitobiose > GlcNAc ≫ GalNAc

zone. S1-type or anti Langmuir isotherms cause an opposite effect [16]. The most other classes of isotherms indicate complexer shaped mass transfer zones.

The interactions between glycoconjugates and lectins are of increasing interest because of their biological function and their technical application. Lectins are proteins able to bind reversibly to carbohydrate structures. Due to the specificity to carbohydrates, lectins are used as ligands in affinity purification of glycoconjugates, a diverse group of substances where carbohydrate chains are attached to either proteins or lipids [17]. Glycoconjugates are involved in different cellular processes such as cell differentiation, immune functions like the recruitment of white blood cells [19] and inter- and intracellular activities [18]. Additionally, glycoconjugates can be seen as the third system to decode information in organismens beside the nucleic acids and proteins [18,20,21] so that they will be of pharmaceutical interest [22–24]. Because of this reason the affinity purification of glycoconjugates with lectins is of increasing importance on analytical as well as on technical scale.

The investigation is confined to a model system containing the two different lectins ConA and wheat germ agglutinin (WGA) (Table 1, Fig. 1) but the principles discussed herein are broadly applicable to other lectins and in more general to other proteins, because most protein products have biological activities or functions which are commonly dependent upon recognition of unique molecular features (e.g. reactive sites, antigenic epitopes or receptor binding domains) in dynamic interaction with other biomolecules.

ConA is a 108 kDa seed protein from Jack bean (*Canavalia ensformis*) first isolated by Sumner in 1919 [25], while WGA is a 36 kDa molecular weight protein and was being isolated from wheat germ (*Triticum vulgaris*) in 1972 [26,27]. These two lectins are frequently used in affinity chromatography and find application in the isolation of polysaccharides and glycoproteins [28–30] or in monitoring of glycosylation patterns of proteins [31]. ConA is a tetramere and got homologous binding sites on each subunit with an affinity to the following monosaccharides with decreasing affinity in the sequence methyl-α-D-mannose > α-D-mannose < α-D-glucose. WGA emerges in dimeric form with two independent binding sites for *N*-acetylglucosamin (GlcNAc) and *N*-acetylneuraminic acid (NeuAc) [32,33].

With ConA and WGA two lectins of different molecular weight and shape (Fig. 1) were selected to notice differences resulting from the size and diffusion behaviour.

Fetuin is a plasma glycoprotein widely distributed in mammals and involved in the acute-phase response [34]. Bovine fetuin, a species homologue to the human plasma protein α_2HS-glycoprotein, can be obtained from fetal bovine serum with a one-step affinity purification at WGA-affinity adsorbents [35]. The monosaccharide composition of the bovine fetuin glycostructure for Gal:Man:GlcNAc:GalNAc is 13.2:11.0:15.5:2.6 [36] and 12.4:9.6:17.2:2.7 [37], respectively.

The FAD-dependent enzyme glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC1.1.3.4) from the fungi *Aspergillus niger* or *Penicillium* catalyses the oxidation of β-D-glucose using molecular oxygen and releasing hydrogen peroxide. It is widely used in technical applications like the determination of glucose in body fluids, the removing residual glucose and oxygen from beverages and foodstuffs or immobilized on gold surfaces in biosensors [38]. The molecular properties and engineering parameters like the diffusion behavior [39] are well investigated. The holoenzyme is in general a dimere of identical 80,000 g/mol subunits. The monomers of glucose oxidase subunits are linked by the non-covalent bond. This enzyme contains four forms of subunit: monomer, dimer, trimer, and tetramer, but only dimer and trimer possess enzymatic activity. During glucose oxidase denaturing, monomers assemble into dimer, trimer, or tetramer. The redistribution behavior depends on the enzyme concentration and the nature of the medium [40].

2. Experimental

2.1. Materials

The lectins ConA (type V) and WGA and the glycoprotein fetuin, as well as sodium borohydride, Bradford reagents, methyl-α-D-mannopyranoside and *N*-acetyl-D-glucosamine were obtained from Sigma (Munich, Germany). Pentaethylenehexamine (PEHA) and glutaraldehyde were purchased from Fluka (Steinheim, Germany), 3-mercaptopropyltrimethoxysilane, 1,4-butane-diol-diglycidyl

ether, pyridine, 2,2,2-trifluoroethanesulfonylchloride were delivered from Fluka (Buchs, Switzerland) and 1-chloro-2,3-epoxypropane came from Aldrich (Poznan, Poland). The enzyme glucose oxidase is available at Serva Electrophoresis (Heidelberg, Germany) and the Micro BCA protein assay reagent kit is distributed by Pierce (Rockford, IL, USA). All chemicals and solvents were of analytical reagent grade.

2.1.1. Support matrices

The chromatographic support Toyopearl AF-Tresyl-650 M (bulk) a methacrylic polymer was obtained from TosoHaas (Stuttgart, Germany). The supplier provides following data: particle size 65 μm, pore diameter 1000 Å, tresyl groups 80 μmol/g. Eupergit C was a gift from the company Röhm in Darmstadt, Germany. The matrix is a copolymer of methacylamide *N*,*N*′-methylene-bis(methacrylamide) glycidyl methacrylate allyl glycidyl ether and methacrylamide containing oxirane groups. The matrix is supplied in form of dry beads and shows a water uptake of approximately 3 mL/g support. According to the supplier it is stable for at least 8 months at <−15 °C. The silica gel XWP-P005 was purchased from the enterprise Grace in Worms, Germany. The preparation of cellulose matix was described elsewhere [41].

2.2. Methods

2.2.1. Surface modification of silica based matrix

Irregular broken silica particles were covered with over molecular sieve dried toluene, before 3-mercaptopropyltrimethoxysilane was added. The mixture was heated up to 90 °C for 1.5 h. The support and reagents were filtered, extracted 4 h with acetone and dried at 80 °C. The product was covered with water and reacted with 1,4-butanediol-diglycidyl ether. The dried material was activated with 2,2,2-trifluoroethanesulfonylchloride under argon atmosphere and stored under HCl, pH 3 [42,43]. The reaction diagram is depicted in Fig. 2 and properties of the support are summarized in Table 2.

2.2.2. Surface modification of cellulose based matrix

2.2.2.1. Preparation of cellulose-based matrix Granocel-4000. Cellulose matrix Granocel-4000 was prepared by saponification of diacetylcellulose by the procedure described previously [41]. Gel of regenerated cellulose was cut mechanically and fractionated by sieving. The fraction of particle size of 160–315 μm was used for further modification. It was determined by inverse gel-permeation chromatography of the dextrans that the pores of Granocel-4000 are accessible to macromolecules of molecular weights up to approximately 2×10^6 g/mol with a specific volume of 1.6 mL/g.

2.2.2.2. Immobilization of pentaethylenehexamine (PEHA) spacer. Fifty grams of sucked cellulose was suspended in the solution containing 46 mL of 2% (w/w) NaOH and 2.5 mL PEHA. Reaction mixture was heated till 50 °C and 3.1 mL of epichlorohydrine and also sodium borohydride $NaBH_4$ was added. The reaction mixture was stirred for 3.5 h at 50 °C. The product was washed with 0.5 mol/L NaOH and water. This synthesis pathway is shown in Fig. 3.

The primary amino groups were determined by desamination with sodium nitrite. Therefore, aminated cellulose was heated at 70 °C for 7 h in the solution (30 mL per g of cellulose) containing 0.1 mol/L $NaNO_2$ and 0.2 mol/L acetic acid. After desamination the cellulose was thoroughly washed with water and the nitrogen content was determined by Kjeldal method. The nitrogen content of primary amino groups was calculated as a difference of nitrogen content in cellulose before and after desamination.

2.2.2.3. Activation of aminated cellulose with glutaraldehyde (preparation of PEHA-Cel). Five grams of aminated cellulose was mixed with 9.4 mL 0.05 mol/L phosphate buffer (pH 8.5) and 2.7 mL of 25% (v/v) glutaraldehyde (pH was regulated with NaOH till 9.3). Reaction mixture was stirred at room temperature for 2.5 h. After reaction, activated cellulose was filtrated and washed with 0.05 mol/L phosphate buffer (pH 7.5).

2.2.3. Immobilization of lectins

The immobilization procedure of the lectins to the silica, cellulose and Toyopearl support was accomplished in 0.5 mol/L phosphate buffer, pH 8, containing 0.5 mol/L of NaCl.

In the case of Eupergit C support the immobilization of ConA molecules took place in HEPES buffer pH 8, both buffers contain Ca^{2+}, Mn^{2+} and Mg^{2+}-ions as well as methyl-mannose. The immobilization yield for all coupling reactions was measured by the determination of unbound lectin concentration in the supernatant. The end-capping of excessive

Table 2
Physical and chemical properties of investigated supports

Name	Abbreviation	Mean pore diameter (Å)	Particle diameter (μm)	Density (g/cm^3)	Specific surface area (m^2/g)	Spacer	Functional group
Modified Silica XWP-GEL P005	Silica	500	84	2.18	78	12 atoms	Tresyl
Toyopearl AF-Tresyl-650 M	Toyopearl	1000	65	1.32 [59]	42	none	Tresyl
Modified Granocel-4000	PEHA-Cel	Exclusion limit (dextrans) 2×10^6 Da	200–315	n.d.	n.d.	18 atoms	Aldehyde
Eupergit C	Eupergit	n.d.	200	n.d.	n.d.	none	Oxirane

n.d.: not determined.

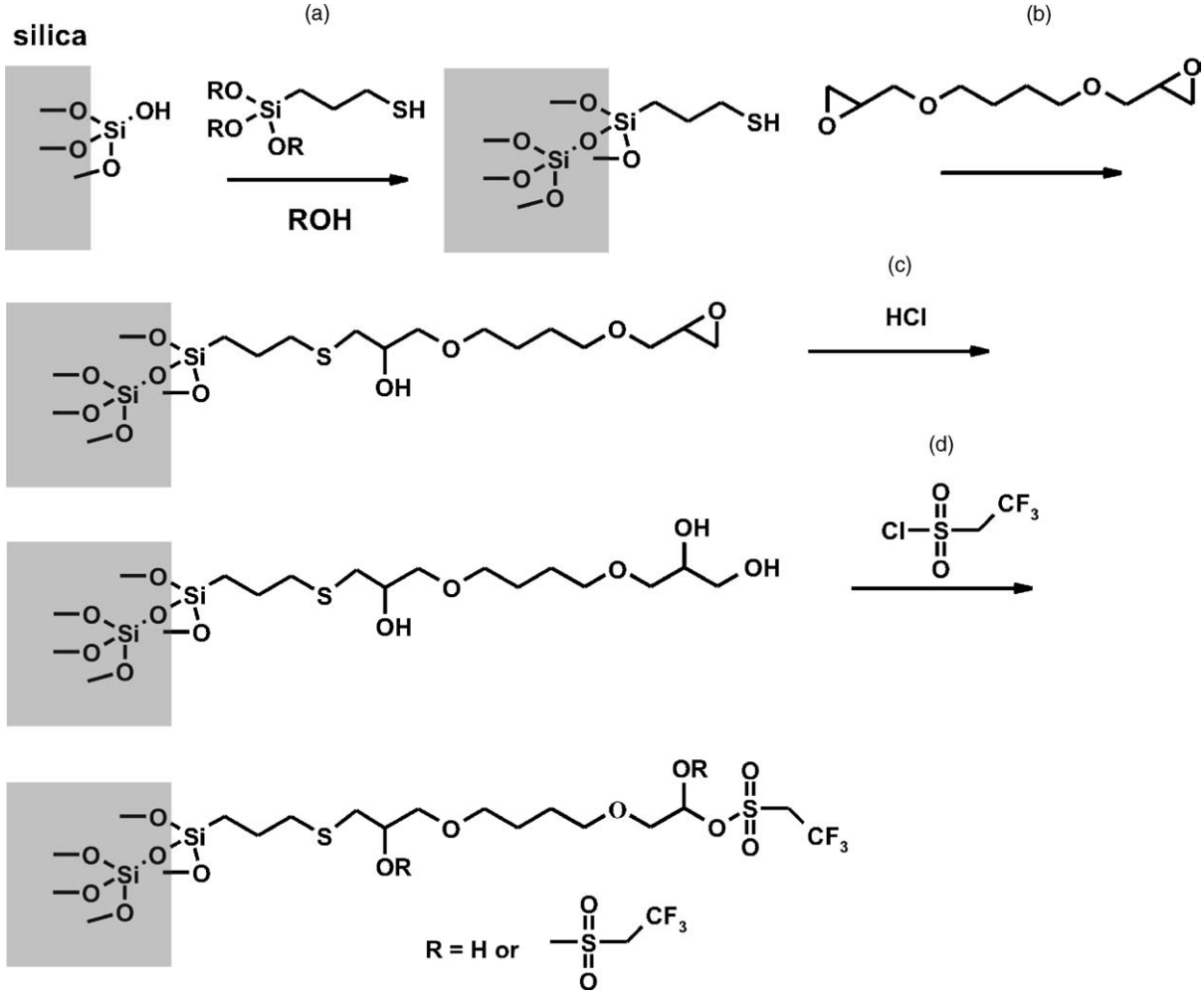

Fig. 2. Synthesis pathway of the modified silica support: (a) silanization of silanol groups on a silica surface with 3-mercaptopropyltrimethoxysilane; (b) reacting with the spacer molecule 1,4-butane-diol-diglycidyl ether; (c) epoxide ring opening with hydrochloric acid; and (d) activation of hydroxygroups with 2,2,2-trifluoroethanesulfonyl-chloride.

functional groups was carried out with 0.5 mol/L Tris buffer. For the cellulose based adsorbents the unreacted aldehyde groups were blocked with $NaBH_4$. 1 g of the support was immersed into 2 ml of 0.1 mol/L phosphate buffer (pH 7.4) with 2 mg $NaBH_4$ and kept for 1 h at 4 °C with subsequent washing.

2.2.4. Protein quantification

BCA protein assay was accomplished in microtiter plates with a linear working range of 2–40 μg/mL protein concentration. 150 μL of the reagent was added to 150 μL of unknown sample, incubated at 37 °C for 2 h and detected at a wavelength λ of 550 nm in a Spectra microplate reader (Tecan, Crailsheim, Germany).

The Bradford method shows good linearity between 2 and 10 μg/mL. Detection occures at λ = 595 nm in the microplate reader. The method was employed, when reducing agents are present that disturb the BCA assay (e.g. carbohydrates, Mn^{2+}). The standards were prepared of the same proteins that are to be determined.

2.2.5. Kinetics of ligand immobilization

The decrease of lectin concentration in the supernatant over the time was measured in a syringe equipped with a filter. In defined time intervals ranging from 0.5 to 400 min droplets of the supernatant were pressed out. 0.1 mL were applied to determine the lectin concentration. The reaction occured at room temperature and the reaction mixture was well shaken. 20 mg lectin was offered to 400 mg weighted dry support (Toyopearl and Eupergit) or adequate amounts of wet support (1 mL silica and 1 g of sucked PEHA-Cel) were used to obtain comparable results. The reaction took place in 9 mL of 0.5 mol/L phosphate buffer. The influence arising from the removal of lectins from the reaction mixture for the detection of the immobilization kinetics was neglected.

2.2.6. Immobilization and adsorption isotherms (binding curves)

The binding capacity of immobilized lectin was determined with the glycoproteins glucose oxidase (GOD) for ConA adsorbents and fetuin for WGA adsorbents. For ConA

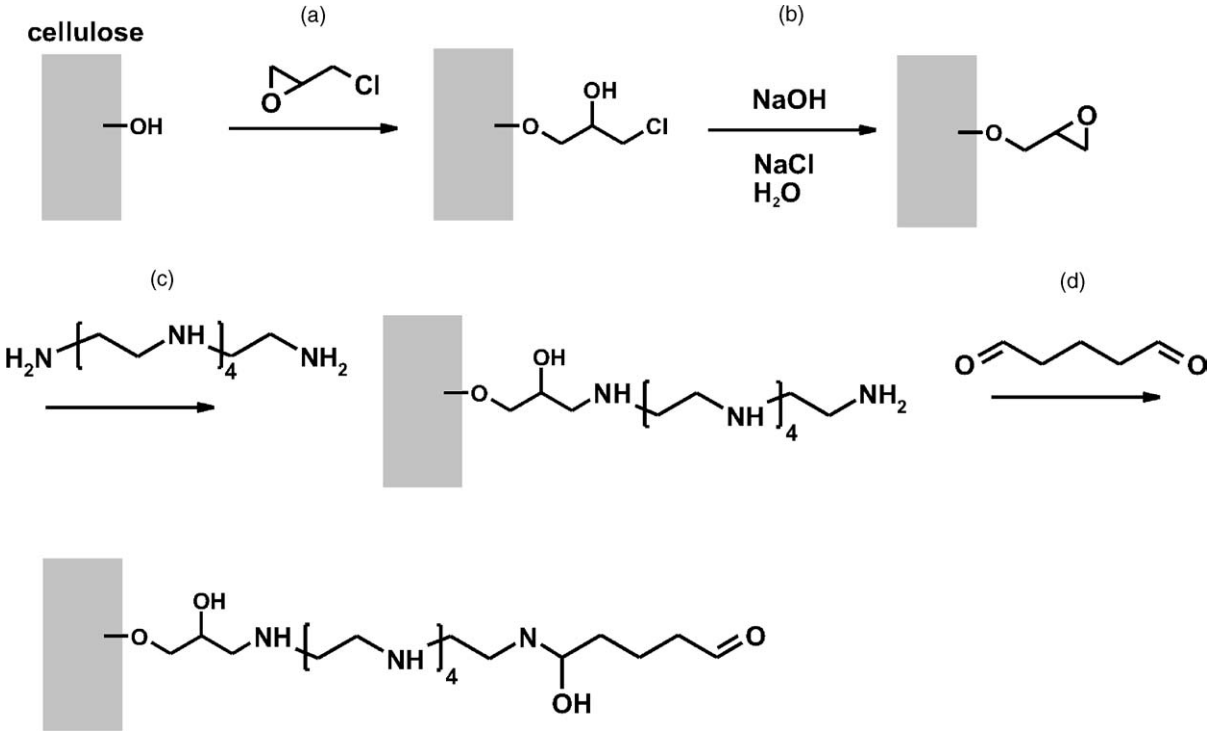

Fig. 3. Synthesis pathway of the modified cellulose support: (a) surface reaction of the cellulose matrix with 1-chloro-2,3-epoxypropane; (b) formation of reactive epoxide with NaOH; (c) attachment of the spacer pentaethylenehexamine (PEHA); and (d) activation with glutaraldehyde.

acetate buffer pH 6 containing 1 mmol/L Ca^{2+}, Mn^{2+} and Mg^{2+} was choosen as adsorption medium. The adsorption of fetuin to WGA occurred in 0.01 mol/L phosphate buffer pH 7.2. Known amounts of glycoproteins with rising concentrations were added to equal amounts of adsorbent and the partition equilibrium was determined by measuring the solute concentration in the liquid phase of the biphasic mixture with known total concentration.

2.2.7. Determination of non-specific adsorption

One hundred milligrams of dry support was endcapped with Tris buffer for 24 h. Variing amounts of glycoprotein solution (GOD and fetuin) were added to the deactivated support. After 30 min the support was washed three times with 5 mL of water and filled up with 1 mL H_2O. 1 mL of BCA protein test solution was added. The colour change in the supernatant is proportional to the non-specific adsorbed protein concentration. Detection occurred at $\lambda = 550$ nm against appropriate standards (linear working range from 0.01 to 0.3 mg/mL of the relevant glycoprotein).

2.2.8. Binding capacity measured in packed columns

One milliliter of adsorbent was filled in a glas-column with the dimensions of 50 mm length and diameter of 7 mm. The flow rate was 0.8 mL/min. ConA adsorbents were washed with acetate buffer pH 6 containing 0.1 mol/L NaCl and 1 mmol/L Ca^{2+}, Mg^{2+} and Mn^{2+}. Desorption was accomplished with 0.1 mol/L methylmannose in acetate buffer. 2 mL of 10 mg/mL GOD solution was applied to the column.

2.2.9. Affinity separation with defined protein mixtures

The purification of fetuin was accomplished with 1 mL Toyopearl-WGA adsorbent, using a 0.01 mol/L phosphate buffer, pH 7.2. The competitive sugar *N*-acetylglucosamine (0.3 mol/L) was employed for desorption. The collected fractions were lyophilized and analyzed with sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). The protein mixture investigated for adsorption specifity contained each 1 mg/mL GOD, BSA and fetuin.

2.2.10. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SDS–PAGE was carried out by the method of Laemmli [44] using a precast Ready Gel 10% Tris–HCl from Bio-Rad developed in a Mini-PROTEAN 3 Cell (Bio-Rad, Munich, Germany). The protein solutions were reduced in 62 mmol/L Tris–HCl buffer pH 6.8 containing 2% SDS and 5% mercaptoethanol at 95 °C. Electrophoresis was driven out at a constant voltage of 200 V for 35 min using an electrophoretic buffer pH 8.3 of Tris-glycine containing 0.1% SDS. The gels were stained with Coomassie brilliant blue solution.

2.3. Protein structure visualization

The data files of the protein structure determination were obtained from the Protein Data Bank [45] and are visualized with the program Deepview/Swiss pdbViewer 3.7 [46]. The picture of WGA is based on the data file 1WGA.pdb [47], of ConA on 5CNA.pdb [48], of the GOD monomer

on 1CF3.pdb [49] and that of the GOD dimer on 1GPE.pdb [49].

3. Results and discussion

3.1. Immobilization of lectins onto the support

The main step in the preparation of the adsorbents is the covalent attachment of lectins to the support. In this respect the kinetics of immobilization and the ligand density which results in surface coverage of the adsorbents were examined and the features of the prepared affinity adsorbent were analyzed.

3.1.1. Kinetics of lectin immobilization

The time required to immobilize the lectins onto the activated support (Fig. 4) is an important parameter in the adsorbent preparation for large scale purification. It depends mainly on the coupling chemistry. The lectin reaction via tresyl groups is rapid and immobilization yields are high. That could be demonstrated for silica XWP-gel P005 and Toyopearl AF-Tresyl-650 M (Table 2). Generally a high pH-value favors the immobilization via tresyl groups [50,51]. At pH 8 the reaction is complete after 30 min. The lectins are linked via amino groups, which are mostly located in lysin amino acid.

Aldehyde groups of the cellulose support Granocel-4000 react with the ligand forming a Schiff's base linkage. The coupling reaction is finished after 3 h. The slowest immobilization reaction occurs between the lectin and the oxirane groups of the Eupergit C support. Coupling reactions with Eupergit support are recommended to persist at least 12 h. Nakamura et al. described a negative effect of prolonged coupling times due to multipoint attachment for the coupling of soybean trypsin inhibitor and certain antibodies [50]. In contrast to Wilchek and Miron, who showed that multipoint attachment does not occur under their experimental conditions. They conclude that the immobilization result in the binding of a protein through one or two surface residues that are sterically exposed [52].

Obviously there is a difference in coupling kinetics between a voluminous molecule ConA (108 kDa) and the smaller one of WGA (36 kDa). The small ligand WGA reacts faster than the bigger one with all supports because of a better mobility and therefore accessibility (Fig. 4). In Table 3

Table 3
Features of the immobilization kinetic

	Lectins	Immobilization rate[a] (mg/min)	$t_{(1/2)}$ (ln–fit) (min)	$\Delta_{(ConA-WGA)}$ (min)
Silica	ConA	1.6	0.01	
	WGA	11.0	0.01	0.00
Toyopearl	ConA	0.7	33.35	
	WGA	1.3	3.93	29.42
PEHA-Cel	ConA	0.1	80.30	
	WGA	0.2	43.35	36.96
Eupergit	ConA	0.2	459.10	
	WGA	0.3	46.81	412.29

[a] Linear range at the beginning.

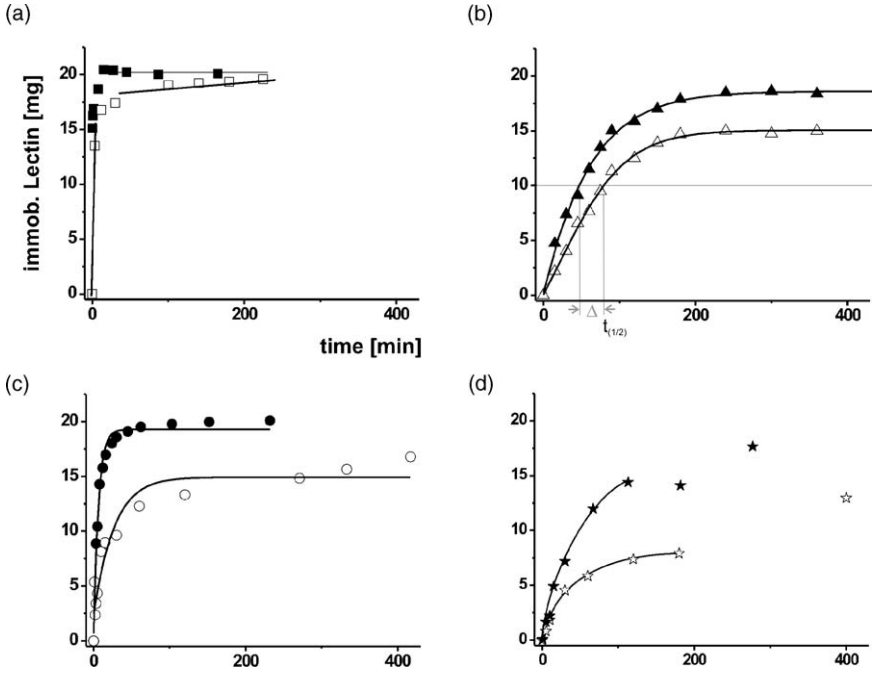

Fig. 4. Immobilization kinetics of wheat germ agglutinin (full) and concanavalin A (light symbol) to: (a) modified silica XWP-P005; (b) modified cellulose Granocel-4000; (c) Toyopearl AF-Tresyl-650 M; and (d) Eupergit C. (b) outlines the different kinetics for the unequal ligands (△) at the time were half of the offered ligand is immobilized $t_{1/2}$.

the immobilization rate in mg per min at the beginning of the reaction is illustrated. Whereas the immobilization of WGA with the silica support exceeds the other reactions about one order of magnitude. To reveal the differences in the immobilization reaction, that derive from the nature of the ligand molecule, Δ(ConA–WGA) was calculated (see Table 3 and Fig. 4b). It shows the difference in the time needed for the immobilization of one half of the offered amount. For the ConA reaction the benefit of a spacer molecule becomes apparent. The kinetics of both supports that are equipped with a spacer (silica XWP-P005 and Granocel-4000) are nearly similar, whereas the supports without spacer (Toyopearl and Eupergit) show a decrease in the immobilization speed as well as in the yield of surface coverage of the large molecule ConA with regard to the smaller WGA. Also the comparison of the supports that bear the same active groups indicate the advantage of a spacer for the immobilization of voluminous molecules. The silica support featured with a 12-C-atom spacer reacts faster than Toyopearl (Table 3). Both supports are activated with tresylchlorid.

3.1.2. Ligand density and surface coverage of affinity adsorbents

The following experiments were done to figure out the optimum ligand density. It should be taken into account that a too high ligand density on the particle surface can cause a decrease in accessibility and thus in binding efficiency due to steric hindrance [1]. On the other hand it should be noted that a low ligand density results in low total capacity of the adsorbent. The immobilization results were taken by offering rising amounts of dissolved ligand molecules to equal amounts of support. Results were shown in Fig. 5.

The silica based support can assimilate most lectin onto it's surface because of its high surface area (see Table 2), whereas the smaller molecule WGA is favored. Except of Toyopearl, which can bind equal amounts of ConA and WGA, all supports favor the binding of WGA. Eupergit and PEHA-Cel are nearly similar with regard to the maximum amount of lectins that can be immobilized.

The surface coverage (SC) was calculated according to the equation:

$$\mathrm{SC}(\%) = \frac{m_{\mathrm{lectin}}\, A_{\mathrm{lectin}}\, 100}{m_{\mathrm{support}}\, A_{\mathrm{support}}}$$

where m_{lectin} is mass of immobilized lectin (mg); A_{lectin} is expansion of the lectin (m^2/mg); m_{support} is mass of the support (g); and A_{support} is specific surface of the support (m^2/g).

The area occupied by the lectins (A_{lectin}) was deduced of the threedimensional structure available at the protein data bank (PDB). 1 mg ConA occupies approximately 0.273 m^2 and 1 mg WGA 0.492 m^2.

For the silica-based support the maximum immobilization capacity leads to a surface coverage of 53% with ConA and 94% with WGA. The Toyopearl surface is at the most occupyable with ConA up to 97 and 82% for WGA. The specific surface area of Eupergit and PEHA-Cel are not known.

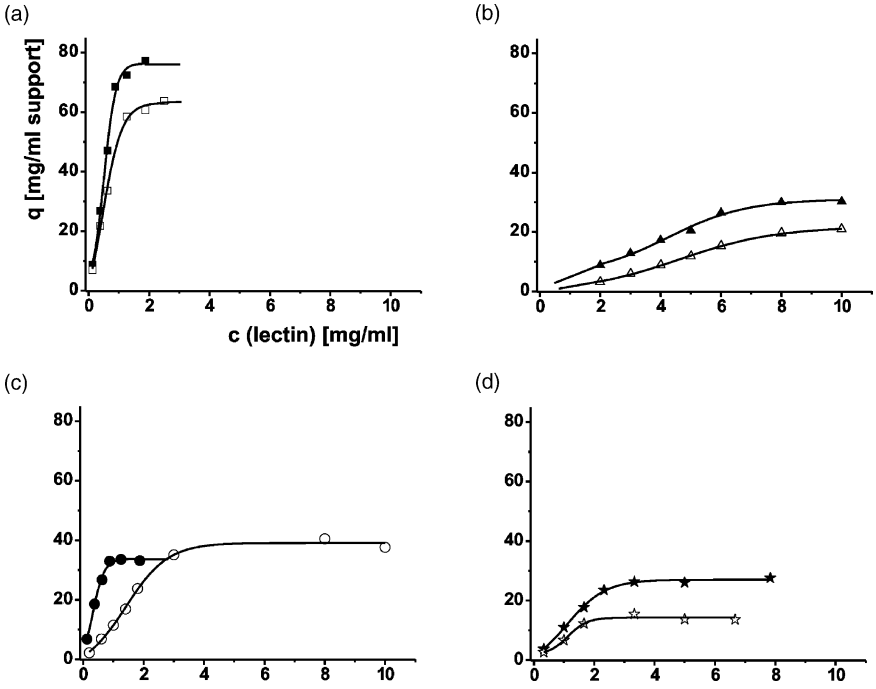

Fig. 5. Immobilization isotherms of wheat germ agglutinin (full) and concanavalin A (light symbol) to: (a) modified silica XWP-P005; (b) modified cellulose Granocel-4000; (c) Toyopearl AF-Tresyl-650 M; and (d) Eupergit C.

3.2. Characteristics of the prepared adsorbents

For the following experiments the adsorbents were covered with moderate lectin density in the way that the immobilization yield is higher than 90% regarding to the offered lectin (preventing high material loss). In the case of silica and Toyopearl 40 mg lectin were offered to 1 g support.

3.2.1. Non-specific adsorption

The ideal matrix contains a completely hydrophilic and non-charged surface. Non-specific adsorption is found to be largely dominated by a combination of hydrophobic and ionic forces [53]. Hydrophobic adsorption can occur at unpolar binding sites of the proteins or the support material e.g. use of long spacer arms may lead to hydrophobic binding sites. Non-specific interactions of ionic character may arise from protein–protein interactions that emerge between polyelectrolytic molecules. Moderate concentrations of salt can suppress ionic effects. Jiang and Hearn [54] observed that the shape of adsorption isotherms of hen egg white lysozyme at Cu^{2+}-IDA Sepharose changed from a Langmuir-type to a shape, which can be fitted with a Freundlich model at higher salt concentrations. They assume that the proteins undergo self-association and multisite attachment. This behavior is similar to the interactions between electrostatic stabilized colloids at higher salt concentrations, but nevertheless the DLVO theory failed to explain the behavior of the protein data in solution at very high salt concentrations [55,56]. Different authors attributed the anomalous stability at high salt concentrations observed to the existence of hydration repulsion resulting from the hydrated counter ions adsorbed onto the protein surface [57]. Out of this reason, the salt concentration has to be decided very carefully.

Within our experimental conditions, a moderate salt concentration of 0.1 mol/L NaCl was used for glycoprotein purification. The determimation of the non-specific adsorption was accomplished in dest. H_2O, because of the sensitivity of the BCA test against buffers. Toyopearl support shows the lowest non-specific adsorption (Table 4).

Table 4
Non-specific adsorption

1 mL support	GOD (mg/ml)	Fetuin (mg/ml)
Silica	1.16	2.10
Toyopearl	0.10	0.15
PEHA-Cel	n.d.	n.d.
Eupergit	0.55	0.33

n.d.: not determined.

3.2.2. Determination of equilibrium constants and adsorption characteristics

Interacting molecules that can be applied for biospecific affinity chromatography usually have K_d values between 10^{-8} and 10^{-3} mol/L. For ConA adsorbents the enzyme glucose oxidase (GOD) was employed. GOD is a glycoprotein (ca. 160,000 Da), which exhibit sugar chains with affinities to ConA. A schematic interaction of GOD to ConA is illustrated in Fig. 6.

By means of the adsorption isotherms, the adsorption capacity and some basic steps of the lectin-carbohydrate interaction can be observed. A more detailed insight into binding mechanisms presents Bisswanger [58]. Isotherms depict the relationship of the amount of the target molecule that is specifically adsorbed and the concentration in solution at equilibrium at constant temperature. The experimental data are shown in Fig. 7. The linearization of these data (plotting q, the amount of adsorbed glycoprotein versus q/c in dependance of the concentration) provides q_{max} (the maximum adsorption capacity) and K_d (the dissociation constant), which are directly available from the interception with the abszissa and the slope, respectively (Fig. 8). The binding of GOD to ConA consists of two steps. High and low affinity binding sites can be identified. Reasons for this behaviour were discussed in Section 3.2.3. The dissociation constant (K_d) of the lectin-glycoprotein complex and the maximum adsorption capacity (q_{max}) were evaluated for the prepared adsorbents and are summarized in Table 5. The experimentally evaluated maximum adsorption capacity shows a reduction of activity in comparison to the calculated maximum capacity (theoretical occupation of every binding site). The non-specific adsorption can be neglected. In

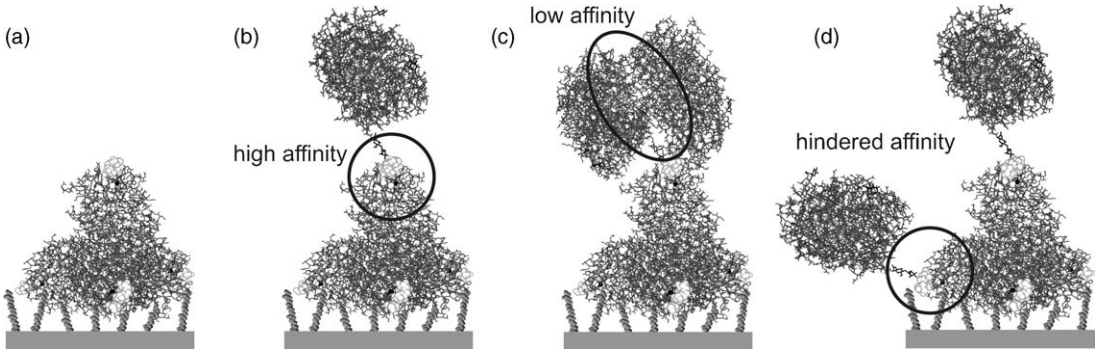

Fig. 6. A proposed interaction of glucose oxidase (GOD) with ConA-affinity adsorbents, depending on the concentration of glucose oxidase: (a) ConA immobilized onto a surface; (b) interaction between immobilized ConA and a GOD monomer at lower concentration; (c) interaction between immobilized ConA and a GOD dimer at higher concentrations; and (d) occupation of hindered binding sites of ConA at higher GOD concentrations.

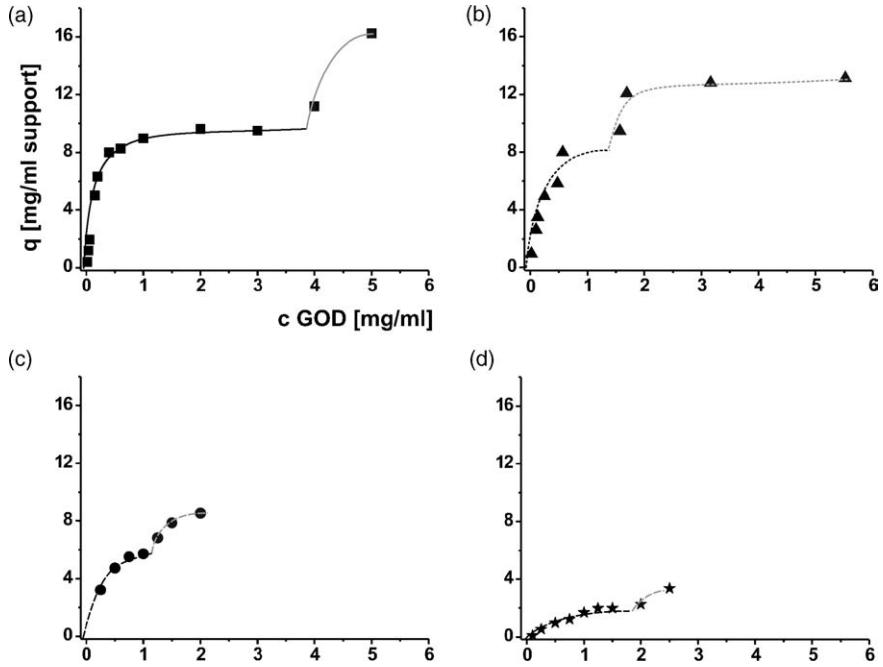

Fig. 7. Adsorption isotherms of glucose oxidase to affinity adsorbents with ConA immobilized onto different supports: (a) modified silica XWP-P005; (b) cellulose Granocel-4000; (c) Toyopearl AF-Tresyl-650 M; and (d) Eupergit C.

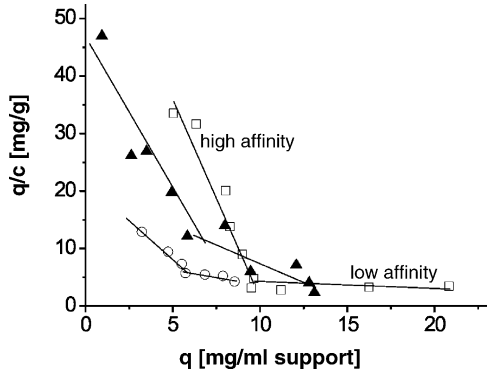

Fig. 8. Linearized adsorption isotherms of glucose oxidase to affinity adsorbents with ConA immobilized onto different supports with concentration depending binding characteristics: (□) modified silica XWP-P005; (▲) cellulose Granocel-4000; and (◯) Toyopearl AF-Tresyl-650 M.

Table 5 the K_d-value was calculated disregarding the different affinities.

The adsorption capacities of the cellulose-based and the silica-based adsorbents are q_{max} = 13.2, and 17.7 mg/mL, respectively which demonstrate a better performance compared to the commercially available polymer-prepared adsorbents Eupergit and Toyopearl (q_{max} = 7.10, and 10.7 mg/mL, respectively). The binding of GOD to the immobilized ConA molecule does not follow the law of Langmuir adsorption. Every isotherm shows an irregularity in its curve progression. The interpretation of these curves provide information of the ConA–GOD interaction.

3.2.3. Characteristics of the specific binding between ConA and GOD

The adsorption isotherms of GOD at a ConA-support (affinity isotherms) can be classified according to Giles et al. as L3- and L4-Isotherms [15]. Over the investigated concentration range, they cannot be mathematically described by a Langmuir isotherm. Fig. 8 illustrates the linearization of the adsorption isotherms. The data exhibit a high and a low affinity range of the GOD adsorption to affinity adsorbents with immobilized ConA on different supports. At lower concentrations of GOD only high affinity binding occures. Low affinity binding takes place after an equilibrium of GOD to the

Table 5
Interaction studies of the affinity adsorbents

Support	K_d (mol/L)	q_{max} (mg) (experimental) per mL support	q_{max} (mg) (calculated) per mL support	Non-specific adsorption GOD (mg/mL)
Silica(ConA) - GOD	3.6×10^{-6}	17.7	24.7	1.16
Toyopearl(ConA) – GOD	4.1×10^{-6}	10.7	13.9	0.10
PEHA-Cel(ConA) – GOD	2.2×10^{-6}	13.2	23.5	n.d.
Eupergit(ConA) – GOD	0.2×10^{-6}	7.10	11.3	0.55

n.d.: not determined. K_d is a mean of the high and low affinity binding sites. q_{max} (experimental) is determined in batch experiments and compared with the theoretical maximum capacity q_{max} (calculated). The non-specific adsorption is listed as useful information.

Table 6
Corrected performance of the ConA adsorbents, with respect to the high and low affinity binding sites to GOD

Adsorbent (1 ml)	K_d (high affinity) (mol/L)	K_d (low affinity) (mol/L)	q_{max} (high affinity) (mg/mL)
Silica(ConA)	8.9×10^{-7}	2.0×10^{-4}	10.3
Toyopearl(ConA)	2.3×10^{-6}	1.4×10^{-5}	8.1
PEHA-Cel(ConA)	1.3×10^{-6}	8.3×10^{-6}	9.7
Eupergit C(ConA)	1.2×10^{-5}	1.6×10^{-4}	4.6

Table 7
Comparison of static and dynamic adsorption capacities q_{max}

Support	q_{max} (calculated) (mg)	q_{max} (experimental) batch (mg)	q_{max} (experimental) dynamic perform (mg)	Usable capacity q under dynamic conditions. In percentage of q_{max} (calculated)
PEHA-Cel (ConA)	23.5	9.7	4.8	20
Toyopearl (ConA)	13.9	8.1	2.9	21

high affinity binding sites was reached. For affinity separation processes only the high affinity binding sites are of interest. Consequently the K_d values must be calculated without the adsorbed amounts in the low equilibrium concentration range (Table 6). Also the capacity of the adsorbents have to be adjusted.

A possible explanation for the different adsorption behaviour could be:

(1) The existence of adsorption sites of different accessibilities, e.g. the orientation of the immobilized ConA exhibits binding sites that are either well accessible or close to the surface (Fig. 6d). The hindered binding sites were occupied only at higher concentrations after saturation of the favoured well accessible binding sites.
(2) The adsorption behaviour can also be estimated to be a consequence of the GOD conformation. At lower concentrations the monomeric form may prevail in solution and the the recognition domain of ConA interacts with the end-standing mannose groups in the glycostructure of the GOD monomere (Fig. 6b). At higher concentrations dimeres are adsorbed (Fig. 6c). Consequently the low affinity binding does not occur between ConA and GOD but is a result of a GOD monomer interaction.

3.3. Application in affinity separation processes

The aim of the prepared adsorbent is to employ them for selective separations to purify glycocompounds (glycoproteins and lipids).

3.3.1. Binding capacity in packed columns

The binding capacity can be measured in terms of partition equilibrium studies (see Section 2.2.6) or by means of affinity chromatography procedures. q_{max} was determined for PEHA-Cel and Toyopearl adsorbents. The maximum amount of GOD that binds to immobilized ConA in a column under dynamic conditions is lower than in batch experiments, which is lower than the theoretically calculated amount (Table 7).

In the batch experiment both the specific and the non-specific adsorption are detected. Consequently in the column experiments q_{max} is determined by the amount that can be specifically desorbed with a competitive sugar. Applying that procedure the non-specific bound GOD remains on the column and is not detected. 20 mg GOD were applied to the column (refer to Section 2.2.8) that corresponds to the calculated maximum capacity of the supports. Only 20% of the calculated total capacity is of use in dynamic column processes. This should be taken into consideration for applications of affinity separations.

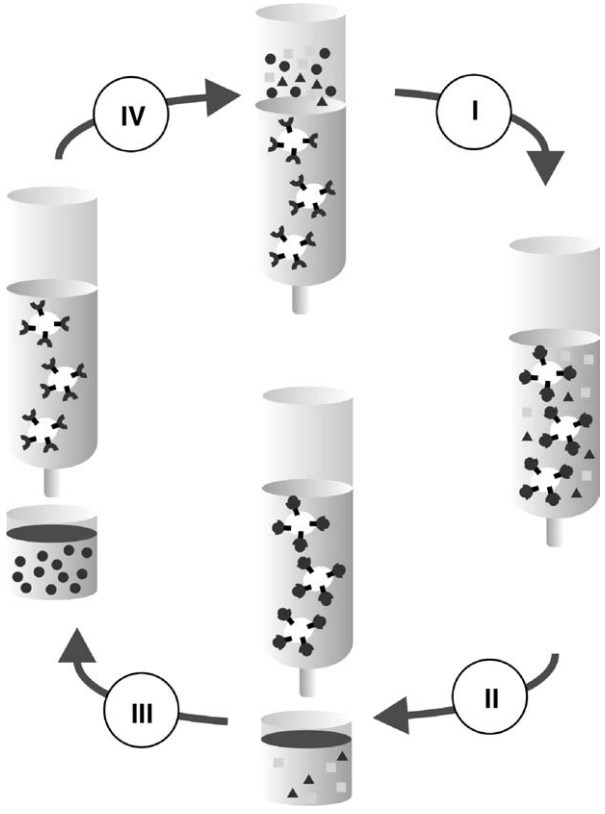

Fig. 9. Affinity separation process containing four steps: (I) adsorption; (II) washing; (III) desorption; and (IV) regeneration. The separation process was used for the isolation of fetuin out of an artificial protein mixture using a WGA-Toyopearl-adsorbent.

3.3.2. Application for protein mixtures

A defined mixture of three proteins GOD, BSA and fetuin was applied to a column packed with Toyopearl-WGA-adsorbent. The proteins have a molecular weight of about 160, 66 and 48.4 kDa, respectively. Fig. 9 illustrates the separation cycle. Four steps were employed to isolate fetuin of a protein mixture. Adsorption, washing, desorption and regeneration were successively accomplished. The adsorption describes the specific interaction of WGA and fetuin. The binding withstands the washing procedure, where unwanted contaminants were removed. The desorption occures with 0.3 mol/L *N*-acetyl-glucosamine in phosphate buffer, which desorbs the specifically bound fetuin in a competitive reaction. After the regeneration, the adsorbent can be used again with the same performance. The adsorbent is applicable for several cycles without significant loss of its activity (data not shown). An SDS–PAGE (Fig. 10) was performed to check the purity of the substances in the collected fractions. In the first row a protein marker was applied. The second lane shows the artificial protein mixture and lane 3 reflects the fetuin standard. The unbound proteins passing the column during the adsorption step, which show no interaction with the ligand are given in lane 4. The purified fetuin is shown in lane 5. Even parts of the fetuin got no retention in the column but most of the fetuin was desorbed. BSA and GOD have no affinity to WGA and have been washed out (lane 5). A WGA affinity adsorbent can be used to purify fetuin out of a model mixture. Cartellieri et al. used a WGA-affinity adsorbens for the one step puification of fetuin from fetal bovine serum [35].

4. Conclusion

Support matrices were investigated and characterized for their feasibility in lectin affinity separation of glycoconjugates. According to the wide influence of the glyco-moieties in biology and medicine, glycosylated bioactive substances and their interactions are of pivotal interest. The initial step of an affinity separation procedure is the preparation of the bio-selective adsorbent and thus the development of immobilization procedures of ligands. This is a critical reaction, because the functionality of the adsorbent may be reduced during this step. Reasons for this may be the chemistry involved, a multipoint attachment of the ligands caused by prolonged immobilization times, steric hinderances due to an overload of the ligands or the choice of inadequate pore size. Hence the investigated supports were analyzed regarding to their immobilization kinetics and their immobilization isotherms. The performance of the tailored supports on the basis of silica and cellulose are better than the commercially available polymeric supports with respect to the adsorption capacity. However, if the non-specific adsorption should be kept to a minimum, Toyopearl support is the best choice. Tresylated supports like the self-prepared silica based supports and the commercial Toyopearl are well suitable for lectin immobilization due to their fast kinetics. The advantage of silica-based support is the possible high amount of ligand that can be immobilized and the large specific surface area and pore size diameter. In the case of using voluminous ligands (ConA) the use of a spacer molecule is proved to be helpful.

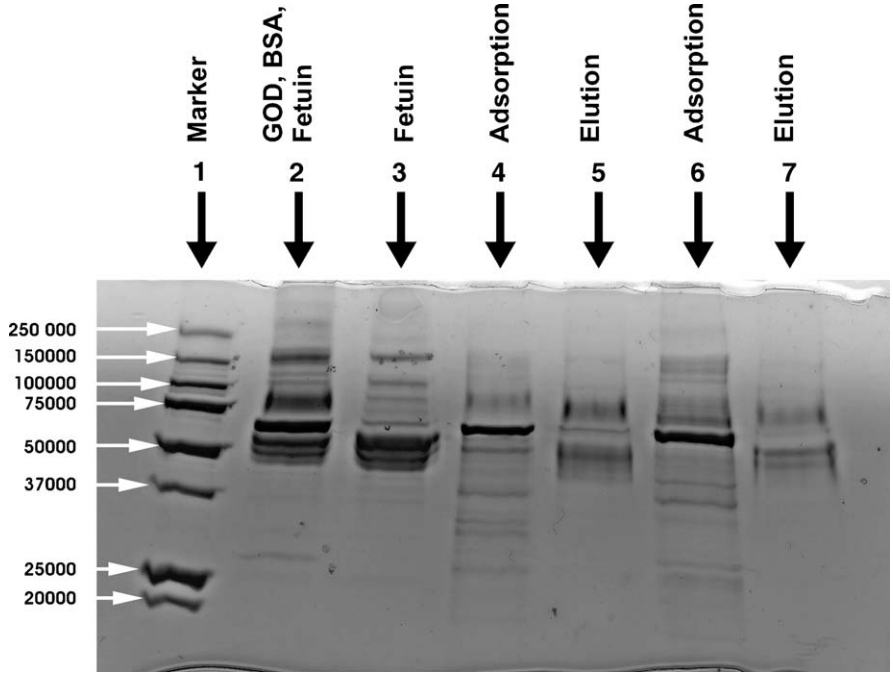

Fig. 10. Analysis of the affinity separation process of fetuin in a protein mixture with WGA-Toyopearl AF-Tresyl-650 M by SDS–PAGE-gel electrophoresis with a precast Ready Gel 10% Tris–HCl (Bio-Rad): (1) precision plus protein standard (Bio-Rad); (2) mixture of glucose oxidase, bovine serum albumin and fetuin; (3) commercially available fetuin; (4) protein passing through the column during adsorption; (5) elution fraction; (6) adsorption fraction of a repeated separation; and (7) elution fraction of a repeated separation.

For a comparative result, the adsorbents were produced identically. Forty milligrams of lectin was offered to 1 g of dry support, respectively. Further the same buffers optimized to the lectin properties were applied. These comparative study clearly indicates that a careful choice of an affinity adsorbent and an application oriented design is crucial for a successful isolation of carbohydrate containing proteins.

Subsequently the prepared adsorbents were evaluated regarding their application in affinity separation processes. For that purpose the adsorption capacity was evaluated. First adsorption isotherms were recorded using batch experiments with the conjugated glycoprotein. Unfortunately non-specific adsorption cannot be excluded. Second the capacity was determined in packed column processes. Overloading the column with adjacent specific desorption prevents the falsification due to non-specific adsorption and the low affinity binding sites are occupied to a lesser extend because of the constant flow in the column. It was shown that only 20% of the theoretical total capacity can be used in lectin affinity chromatography processes.

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