DOI: 10.1002/adsc.200600254

Synthesis of Glyco-Silicas by Cu(I)-Catalyzed "Click-Chemistry" and their Applications in Affinity Chromatography

Mariano Ortega-Muñoz,[a] Javier Lopez-Jaramillo,[a] Fernando Hernandez-Mateo,[a] and Francisco Santoyo-Gonzalez[a,*]

[a] Instituto de Biotecnología, Departamento de Química Orgánica, Universidad de Granada, 18071 Granada, Spain
Fax: (+34)-958-243-186; e-mail: fsantoyo@ugr.es

Received: May 31, 2006; Accepted: September 5, 2006

Abstract: The covalent immobilization of suitable alkyne/azide carbohydrate derivatives on complementarily functionalizated azide/alkyne silica was performed by click ligation througth the Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction of such compounds. The new glyco-silicas have shown to be efficient and valuable bio-selective affinity chromatographic supports for the purification of lectins as well as for the one-pot fluorescent labeling of those proteins. The synthetic methodology is simple, high yielding and flexible, allowing the preparation of tailored glyco-silicas with potential future applications in the inmobilization of other biomolecules.

Keywords: affinity chromatography; carbohydrates; click-chemistry; cycloaddition; fluorescence; glyco-silica

Introduction

Immobilization of biomolecules is a mainstay in biological and related areas with many potential applications such as the characterization of their functions and their interaction with other biomolecules, the application in the analysis and purification of mixtures of biomolecules, and the design of solid-phase based essays or bioactive implant surfaces as the most representative examples.[1] These applications rely on the specific interactions of those biomolecules with other molecules such as the case of antibody-antigen, the carbohydrate-lectin or the enzyme-inhibitor interactions and require that one of the members of the interacting pair be fixed onto an adequate solid support. In the broadest sense, two current immobilization methods are used for the attachment of biomolecules to a surface: non-covalent immobilization based on physical adsorption and direct-covalent immobilization to chemically functionalized surfaces. Microarrays,[2] bionanoparticles,[3] microbeads,[4] biosensor chips[5] and self-assembled monolayers (SAMs)[6] are some of the most valuable tools developed from biomolecule-based surfaces with important applications in the "omic" sciences.

On the other hand, affinity chromatography[7] is one of the most important techniques based on the immobilization of biomolecules onto a variety of solid supports that exploits the various biological interactions mentioned above for the analysis and purification of biomolecule mixtures. Considering the type of supports, two affinity methods are distinguished: low-performance affinity chromatography in which the support is usually a large-diameter, non-rigid gel (such as agarose, dextran or cellulose) and high-performance affinity chromatography (HPAC) that uses small, rigid particles based on silica or synthetic polymers that are capable of withstanding the high flow rates and/or pressures that are characteristic of HPLC systems. The technique has proved to be very flexible allowing a wide variety of applications mainly by the chemical covalent ligation of lectins (lectin affinity chromatography),[8] proteins A and G[9] and antibodies (immunoaffinity chromatography)[10] as the capture biomolecules. In this regard, carbohydrates have been less extensively used as sugar affinity ligands[11] mainly due to the intrinsic difficulties in the synthesis of carbohydrate-containing molecules because of the requirement of orthogonal hydroxy group protection strategies and the challenge of stereoselective glycosidic bond formation. However, recent advances in chemoselective ligation have provided efficient techniques for the covalent immobilization of carbohydrates on a solid surface.[12] The different developed methodologies require a modified surface and a suitable functionalized glycoconjugate with complementary functional groups in order to obtain the desirable bioorthogonality for the grafting. The reactions of sulfhydryl groups with maleimides and disulfides, the Staudinger ligation involving azides and specially-designed

phospine reagents, the Diels–Alder cycloaddition and the Huisgen 1,3-dipolar cycloaddition have demonstrated to be powerful chemoselective reactions that found important applications not only in the construction of immobilized carbohydrate arrays[13] but also in cell-surface engineering and in the synthesis of glycoproteins and glycosylated natural products.[12]

Among the above-mentioned chemoselective reactions, the Huisgen dipolar cycloaddition of alkynes and azides[14] has been recently established as an important synthetic tool under the "click-chemistry" concept[15] especially after the discovery that Cu(I) salts catalyzed regioselectively the ligation of such functionalities at room temperature.[16] Additional benefits of this reaction are the easy introduction of azides and alkynes into a substrate and the stability of such functions that tolerate water and oxygen allowing a modular assembly for individual building blocks. Finally, the triazole formation resulting from the fusion of these functions is irreversible and usually high yielding. All these outstanding features have found huge applications in biomedicinal science, organic synthesis and materials science.[17] In particular, novel functionalized supports based in silica[18] and agarose[19] as well in the covalent ligation of carbohydrates probes to a solid surface[20] for the development of glycoarrays,[21] carbohydrate-containing quantum-dots[22] and sugars-SAMs[23] have been efficiently prepared by this methodology.

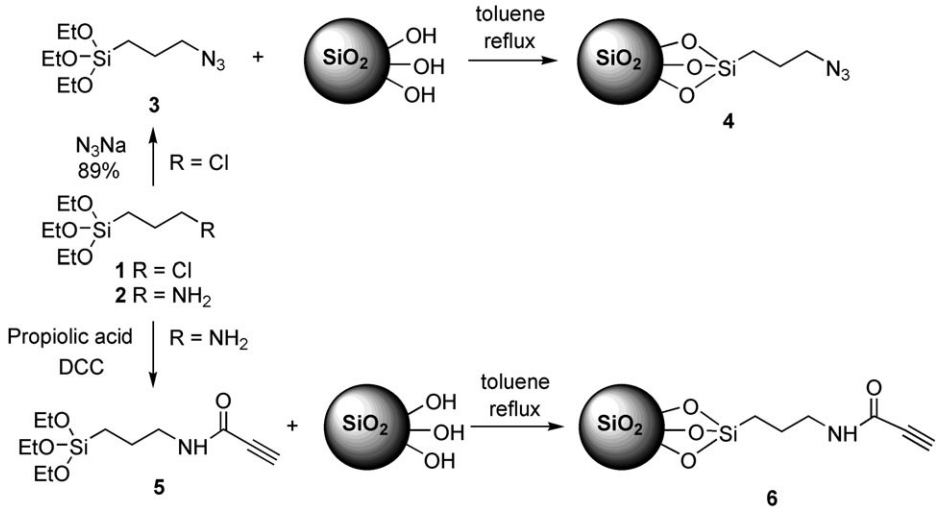
As a chromatographic support, silica is advantageous because its rigid mechanical structure makes it invulnerable to swelling and resistant to large changes in pressure and flow rates without disintegrating or deforming. However, although silica is available in a wide range of pore and particle sizes and macromolecules are adsorbed to its surface, it is not routinely used for immobilization and affinity purification of macromolecules. The strong electrostatic interactions that dominate the process[24] are difficult to tune and the curvature of the silica particles provokes a distortion of the protein's secondary structure that diminishes the catalytic activity.[25] Recent studies have revealed the orientation of BSA adsorbed to silica[26] and demonstrated that the covalent attachment of PEG to lysozyme alters the preferred molecular orientation and makes its adsorption less irreversible.[27] These experimental results provide additional insight into the adsorption process and may have potential interest for the use of silica as immobilization support. As both the modification of the protein and the modulation of its orientation are difficult to achieve, a more feasible approach is the modification of the surface of the silica particles to tune the affinity and specificity for macromolecules. The introduction of organic functional groups on a silica surface to yield modified silicas has received great attention for the development of organic-inorganic hybrid having found applications in chromatography, catalyst and clean technology.[28] In particular methods such as covalent grafting of polymers or coating with hydrophilic polymers have succeeded in passivating the silica surface and conferring specificity. In these cases, coupling agents have demonstrated to play a role in the interaction among the protein and the bed leading to differences in the chromatographic behavior of the functionalized silica.[29] In spite of these facts, silica materials containing covalently bound sugars are scarcer in the literature having found a limited use for boron removal[30] and the isolation and purification of lectins.[31]

With the aim of developing a fast, efficient and inexpensive procedure for the immobilization of carbohydrates, we turned our attention to silica gel as a universal and ready accessible solid scaffold to be functionalized with azido and alkyne functions in tandem with the Cu(I)-catalyzed 1,3-dipolar cycloaddition of such functions as an economical and high yielding regioselective methodology for the grafting of such biomolecules and leading to glyco-silicas. Moreover, in order to evaluate the potentiality of such materials, we also decided to study the application of these materials as affinity chromatography supports for the purification and isolation of lectins based on their specific interactions.

Results and Discussion

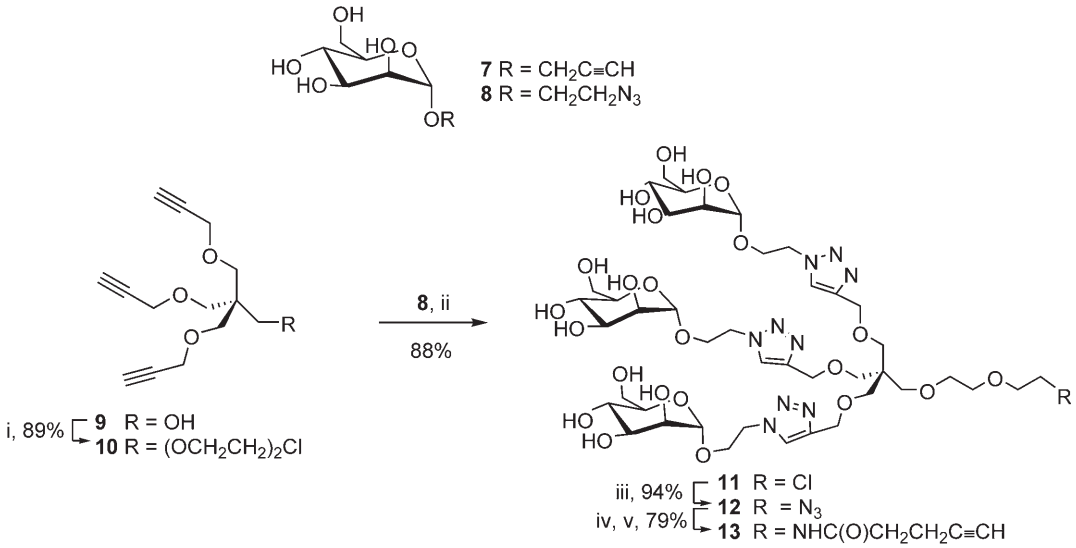
To attain the pursued goals concanavalin A (ConA) was the lectin of choice considering that it is one of the most widely used in carbohydrate-lectin interaction studies. As ConA binds specifically α-D-mannopyranoside (α-D-Man*p*) and α-D-glucopyranosides, we decided to prepared glyco-silicas containing α-D-Man*p*. Surface chemical modification is the first required step of the process leading to the immobilization of biomolecules on a solid support. For our purposes, the chemical functionalization of silica was performed by silanization of activated commercial silica with adequate azide and alkyne derivatives of triethoxysilane that allow the covalent ligation of such derivatives. (3-Azidopropyl)triethoxysilane (**3**)[32] and propiolic acid (3-propyltriethoxysilane)-amide (**5**) were easy prepared from commercial 3-chloro- and (3-aminopropyl)-triethoxysilanes **1** and **2**, respectively, following standard chemical procedures (see Experimental Section). These silane reagents were then reacted with activated silica by a simple refluxing process of a toluene suspension. By this way the azido and alkyne functionalized silicas **4** and **6** were efficiently obtained (Scheme 1).

Considering that the carbohydrate-protein interactions found in nature are multivalent to allow an efficient and specific recognition of such biomolecules,

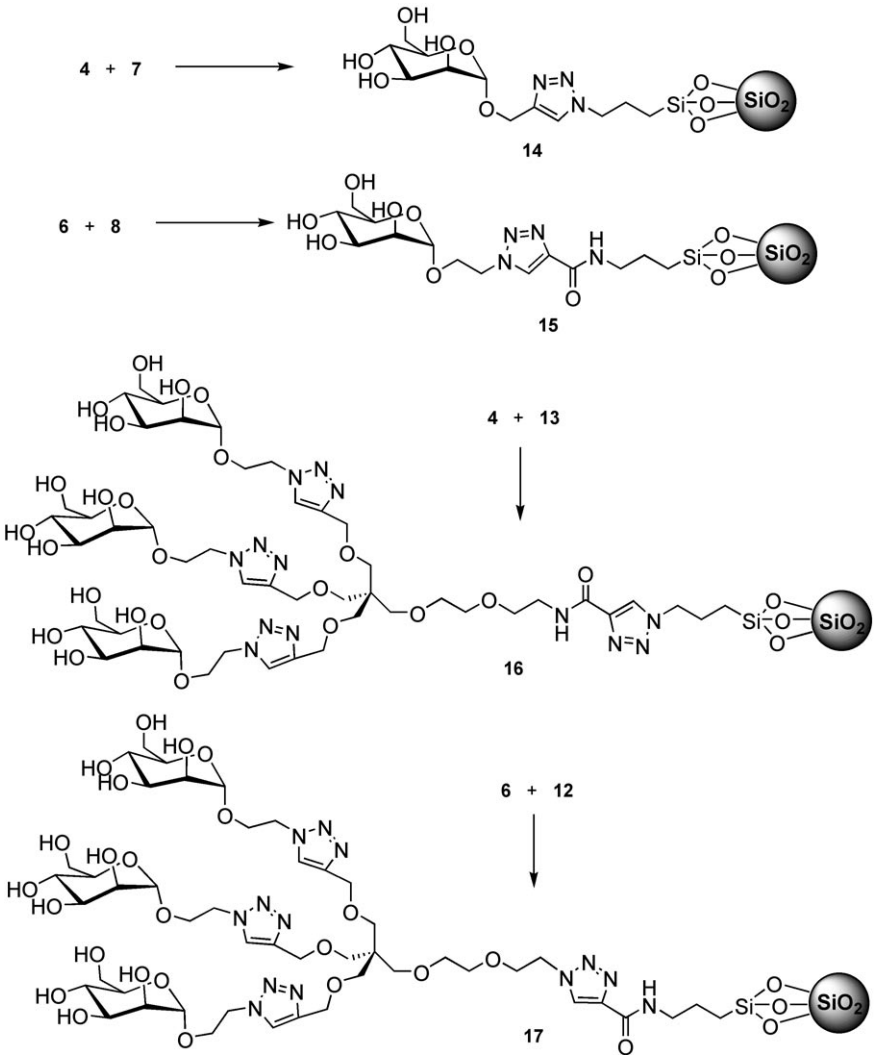


Scheme 1. Synthesis of functionalizated silicas.

the immobilization of not only monovalent but also of multivalent carbohydrate displays over silica was planned by choosing the alkyne and azido monosaccharides **7**[33] and **8**[34] as well as the trivalent dendrons **12** and **13** which are readily obtainable by click-chemistry from the pentaerythritol derivative **9**[35] as depicted in Scheme 2. *O*-Alkylation with an excess of 2-chloroethyl ether[36] gave the trialkynyl derivative **10** suitable for click conjugation with **8**. The reaction was performed in toluene as solvent and using the soluble Cu(I) copper catalyst $(EtO)_3P{\cdot}CuI$ and microwave irradiation as the optimal reaction conditions taking into consideration successful previous results found by us using this 1,3-dipolar cycloaddition reaction in the construction of molecules with diverse architectures.[16c] The Man trivalent glycodendron G1-Cl **11** was thus obtained in excellent yields. Nucleophilic substitution of the chlorine by azide gave the glycodendron G1-N_3 **12** which was further transformed in the alkynyl glycodendron G1-C≡CH **13** by a two-step reaction sequence (hydrogenation followed by acylation with 4-pentynoic acid). Chemical click ligation of the complementarily functionalizated alkyne (**7** and **13**) and azido sugar (**8** and **12**) derivatives to the functionalized silicas **4** and **6** was carried out using the same reaction described above yielding the glyco-silicas **14–17** that were easily isolated by filtration and extensively washed and dried prior to their further utilization (Scheme 3).



Scheme 2. Alkyne and azide carbohydrate derivatives for click-chemistry inmobilization on functionalized silica. *Reaction conditions*: (i) $(ClCH_2CH_2)_2O$, NaOH; (ii) $CuI(C_2H_5)_3P$, DIPEA, toluene, MW; (iii) NaN_3/DMF/80 °C; (iv) H_2, Pd/C. MeOH; (v) $HC{\equiv}CCH_2CH_2COOH$, DCC, CH_2Cl_2.



Scheme 3. Synthesis of glyco-silicas **14–17** by Cu(I)-catalyzed "click-chemistry".

Once the glyco-silicas were synthesized, their ability to bind ConA was evaluated as an adequate methodology to characterize and determine their potential applicability as a chromatographic support. It has been previously proven that affinity chromatography is a valuable tool for the analysis of insoluble systems[37] since hemagglutination inhibition studies (HIA) and enzyme-linked lectin assays (ELLA) (that are the usual techniques for the analysis of carbohydrate-protein interactions) are restricted to assays in solution. The characterization was carried out by packing the glyco-silica in a column followed by saturating this column with a ConA solution in HEPES buffer and then washing with HEPES buffer to remove the unbonded protein. In order to evaluate the strength and nature of the interaction between ConA and the glyco-silicas, the columns were subjected first to a gradient of NaCl to identify unspecific interactions followed by elution with 1 M methyl α-D-mannopyranoside (α-D-Man*p*OMe) to verify the specific Man-ConA interactions. A similar protocol was followed with raw-silica that was thus established as a control assay.

From the results summarized in Table 1 different conclusions can be deduced. First, raw silica appears to be the most effective in retaining ConA (41 % versus 19–34 % in the case of glyco-silicas **14–17**). This fact is remarkable, especially when considering that both silica and ConA (estimated isoelectric point 5.27)[38] are negatively charged and electrostatic repulsions are expected to disfavor the interaction. This unexpected adsorption is not exclusive for ConA but has been previously observed for ferritin (estimated isoelectric point 5.4)[39] and studied in detail for BSA (isoelectric point 4.7)[40] and *endo*-β-1,3-glucanase (isoelectic point 4.4).[41] The adsorption of proteins on a hydrophilic surface under conditions of electrostatic repulsions was predicted by Arai and Norde[42] for *soft* proteins, meaning proteins with low conformational stability which allows structural arrangements

Table 1. Summary of the characterization of glyco-silicas **14–17** and comparison with raw silica.[a]

	Raw silica	Glyco-silicas			
		14	**15**	**16**	**17**
ConA initially trapped by the column (mg)	0.93	0.58	0.45	0.93	0.63
ConA eluted by washing with buffer (mg)	0.32	0.17	0.17	0.45	0.31
ConA retained by the column (mg)	0.61	0.41	0.28	0.48	0.32
ConA retained by the column (%)[b]	41	27	19	34	21
ConA eluted by NaCl gradient (mg)	0.18	0.12	0.07	0.04	0.00
Retained ConA eluted by NaCl gradient (%)[c]	29	29	25	8	0
ConA eluted with α-D-Man*p*OMe (mg)	0.13	0.25	0.21	0.41	0.26
Recovery with α-D-Man*p*OMe (%)[d]	30	86	100	93	81
Silica loading capacity[e]	0.50	0.96	0.81	1.58	1.00

[a] ConA was estimated spectrophotometrically assuming A_{280} 0.1% (=1 g/L) 1.08. Calculations are referred to 1.5 mg of ConA present in the tested sample and 260 mg of raw silica or glyco-silica packed in the column (see Experimental Section).
[b] % = (ConA retained by the column/1.5) × 100.
[c] % = (ConA eluted by NaCl gradient/ConA retained by column) × 100.
[d] % = ConA eluted with α-D-Man*p*OMe/(ConA retained by column − ConA eluted by NaCl) × 100.
[e] Loading capacity means amount of ConA adsorbed specifically by the resin = mg of ConA/g silica

of the molecule upon adsorption and turns the conformation entropy gain into the driving force that overcomes the unfavorable electrostatic repulsions. This hypothesis is in full agreement with the reported reduction of the α-helix content for the BSA molecules adsorbed to silica and might explain the behavior of ConA whose tertiary structure consists of three β-sheets with 50% of the residues in loop regions.[43] However, in this regard, it should be also mentioned that a recent study has demonstrated that *endo*-β-1,3-glucanase behaves as a *hard* protein that deviates from the classical model by being adsorbed on negatively charged surfaces while maintaining its native structural characteristics.

Secondly, the results of Table 1 indicates that the amount of ConA released by the gradient of NaCl is dependent on the nature of the glycoconjugate, being negligible for trivalent silica gels **16** and **17** and significant for the monovalent glyco-silicas **14** and **15** which are similar to raw silica. This result supports earlier observations on the influence of the interaction of ConA with polysaccharides and glycoproteins and its relation with the structural differences of the grafted saccharides.[44] However, regardless of the functionalization, α-D-Man*p*OMe provokes the elution of the ConA attached to the silicas, even from raw silica, although the functionalization of glyco-silicas improves the recovery from 30% in the case of raw silica to 81–100% for glyco-silicas **14–17**, making the synthesized materials suitable for chromatographic applications.

In order to evaluate the potential of the new modified silica beds, we focused on two specific problems that need to be improved despite the myriad of different technologies and work flows currently available in proteomic research: sample protein separation and protein labeling. Among the separation techniques, affinity chromatography has been reported as a powerful technique that can be used as part of the traditional work flow[45] and may circumvent some of the technical disadvantages of two-dimensional electrophoresis. For the particular case of glycomics, glycoaffinity chromatography is a well established approach to separate glycoproteins/carbohydrate pairs[46] and elucidates interactions and specificities.[47] To test the potential of the synthesized glyco-silicas as beds for glycoaffinity chromatography, we selected the glyco-silicas **14** and **16** on the basis of their structure (monovalent *versus* trivalent Man functionalization) and loading capacity and they were compared with raw silica for the purification of the ConA contained in 1 mL of a 3 mg mL^{-1} solution consisting of 0.15 mg mL^{-1} of commercial Con A and 2.85 mg mL^{-1} of a crude extract from plant roots. The experiment is summarized in Figure 1. As expected from the characterization described above, raw silica and the glyco-silica **16** trapped similar amounts of protein from the extract and more than **14**. However, unlike raw silica, the proteins attached to the functionalized glyco-silicas were insensitive to 1.6 M NaCl and sensitive to α-D-Man*p*OMe (see insert in Figure 1). The electrophoresis of the peak (Figure 2) confirmed the success of the purification and demonstrated that a single passage suffices to isolate the ConA present in the extract to a higher purity than the commercial sample. The performance of glyco-silica **16** is remarkable as 98% of the ConA contained in the sample was recovered, 82% being in the peak fraction (Figure 1). To further analyze the influence of the nature of the glyco appendages on the chromatographic properties of the glyco-silicas, a second purification experiment was carried out. The protein mixture consisted in a

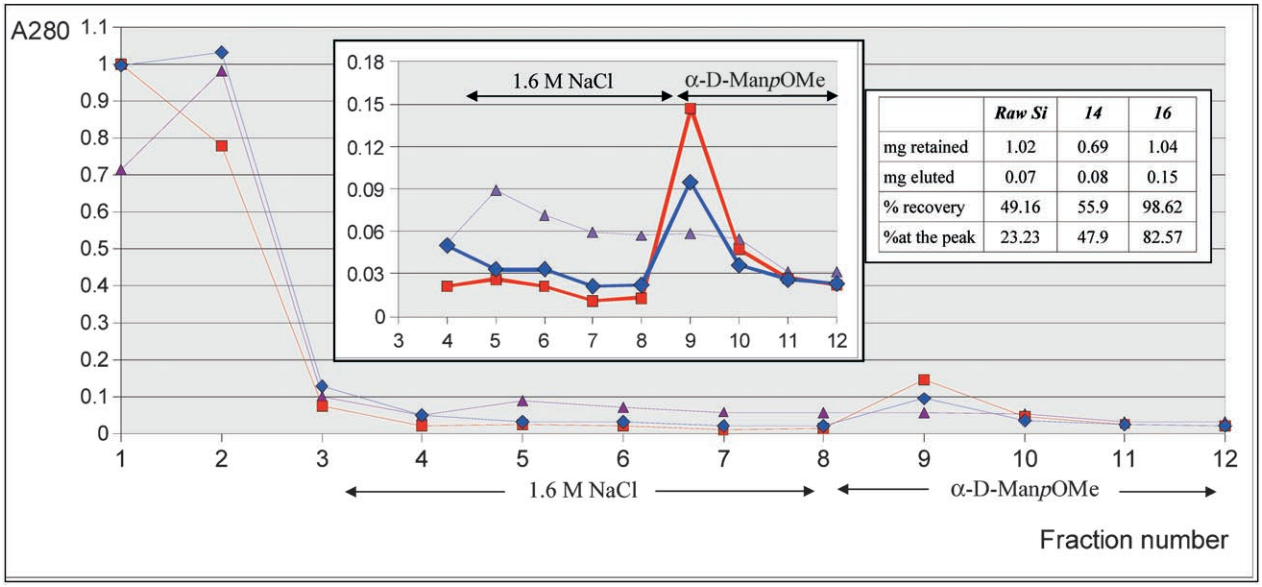


Figure 1. Chromatograms showing the performance of glyco-silicas **14** (◆) and **16** (■) and comparison with raw silica (▲) as glycoaffinity beads for the purification of ConA from a sample contaminated with a crude extract from roots of plants. Insert shows a detail of the effect of ionic strength and α-Man*p*OMe on the bound ConA. The table summarizes the yield of the elution with α-D-Man*p*OMe. *Fraction 1*: unbounded ConA; *Fraction 2*: washing with HEPES buffer; *Fractions 3–8*: washing with 1.6 M NaCl in HEPES buffer; *Fractions 9–12*: elution with 1 M α-D-Man*p*OMe in HEPES buffer.

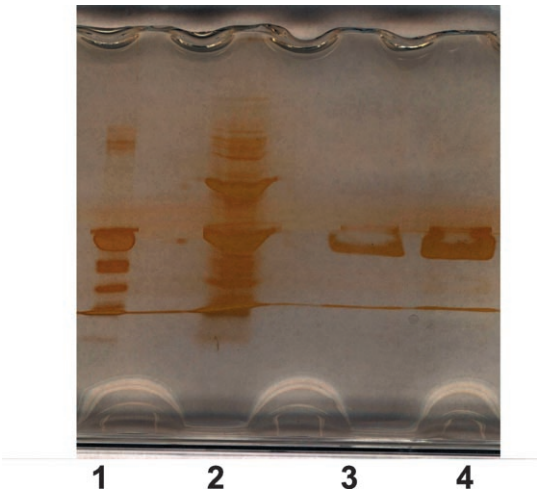


Figure 2. Analysis by SDS PAGE and silver stain of the performance of **14** (*lane 3*) and **16** (*lane 4*) for the purification of commercial ConA (*lane 1*) from a mixture (*lane 2*).

sample of a 1.6 mg mL^{-1} solution containing 0.4 mg mL^{-1} commercial ConA and 1.2 mg mL^{-1} lysate from *Escherichia coli*. As shown in Figure 3, the dendritic glyco-silicas **16** and **17** show better chromatograms yielding sharper peaks while the protein eluted from the monovalent Man-functionalized silica **14** is split into two fractions.

The potential of protein immobilization in a glyco-silica column is not limited to protein purification and identification but is also a valuable approach to modify proteins while preserving the integrity of the carbohydrate recognition domains (CRDs) that are the regions responsible for the interaction with the glyco-silicas. As protein detection in many of the high-throughput systems involved in proteomics is based on fluorescent labeling,[48] it was decided to label ConA with a fluorescent reagent. Among the fluorescent reagents available in the market, those with a benzofurazan skeleton have been widely used in bioscience[49] although their low water solubility has limited their use in protein labeling. However, the fact that 4-nitro-7-benzofurazanyl ethers show improved water solubility and reactivity could solve this limitation and since they are not commercial the synthesis of 4-(2-hydroxyethoxy)-7-nitro-2,1,3-benzoxadiazole (NDB-OCH_2CH_2OH) was carried out following the reported procedure.[50] The labeling of ConA with NBD-OCH_2CH_2OH was effected while the lectin was attached to the glyco-silica **16**. Although it is known that the prevalent structure of ConA found at the water/mica interface is the dimer,[51] the immobilization was carried out at pH 5.2 to force ConA to exist as dimers instead of tetramers, ensuring by this way the maximum protection of CRDs by interacting with the Man units of the glyco-silica. Prior to labeling, the column was equilibrated against phosphate buffer pH 8.4 and the reaction was carried out by recirculating overnight NBD-OCH_2CH_2OH (1 mL of a 7.25 mM solution in buffer phosphate). After washing with HEPES buffer to remove the excess of NBD-OCH_2CH_2OH, the sample was eluted with 1 M α-D-

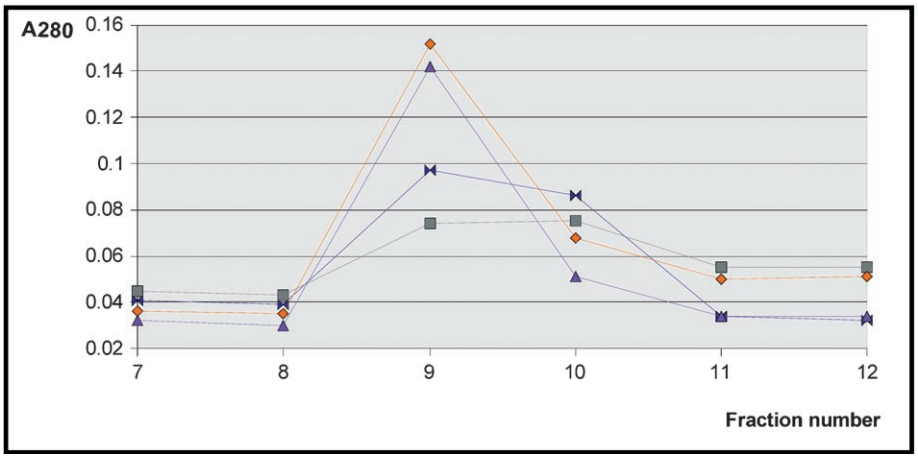


Figure 3. Detail of the chromatograms showing the elution peak resulting from the purification of ConA from a sample contaminated with an *E. coli* extract with the glyco-silicas **14** (►◄), **15** (■), **16** (◇), **17** (▲). *Fractions 7 and 8*: washing with 1.6 M NaCl in HEPES buffer; *Fractions 9–12*: elution with 1 M α-D-Man*p*OMe in HEPES buffer.

Man*p*OMe in HEPES buffer. The rationale behind this buffer exchange and pH shift is that at alkaline pH the reaction takes place at the amino group yielding more fluorescent derivatives[52] while at acidic pH the reaction is slower *via* SH groups[53] which are not present in ConA. The success of the labeling was analyzed by SDS PAGE and visualized with a UV source. As shown in Figure 4 the band corresponding to the labeled protein is visualized when the gel is illuminated with a standard UV transilluminator

Finally, it should be mentioned that the prepared glyco-silicas prove to have a good chemical stability which enhances their potential as affinity adsorbents for the procedures of the separation cycle: adsorption, washing, desorption and regeneration. Thus, the dried glyco-silicas were stored at least for three months at room temperature prior to the packing process without lose of their capacity as affinity supports. Once the columns were prepeared, they underwent eight freeze-thaw cycles for their reuse with maintenance of their performance. In addition, the results were reproducible when the experiments were repeated.

Conclusions

The results reported herein demonstrated that the Cu(I)-catalyzed 1,3-dipolar cycloaddition of alkyne and azides ("click-chemistry") is a general, versatile and efficient synthetic methodology for the immobilization of biomolecules such as carbohydrates, allowing the easy preparation of tailored glyco-silicas. These new materials have shown to be valuable bioselective affinity supports in which the unspecific interactions that make silica unsuitable for affinity chromatography are passivated, turning raw silica into a support with a clear potential in glycomic research.

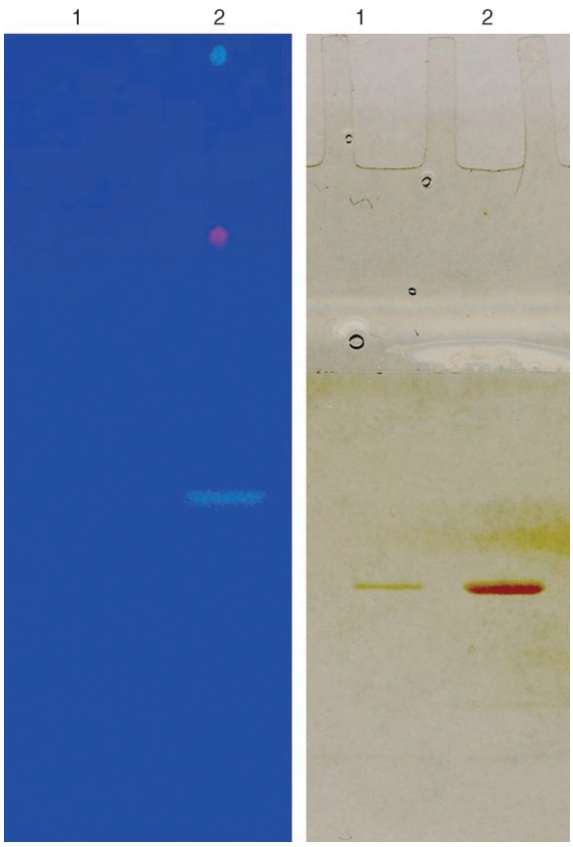


Figure 4. SDS-PAGE of the labeled ConA (*lane 2*) and control (*lane 1*) ConA and visualization with a UV source (*left*) and silver stain (*right*).

The characterization of such materials, performed by evaluating their binding capacity of a suitable lectin (ConA), demonstrated that the structure of the glyco appendages plays a role in the chromatographic properties of those glyco-silicas showing a better perfor-

mance in the case of a dendritic architecture respecting to a monovalent structure. Thus, among the glyco-silicas prepared herein, **16** proved to be the most appropriate for target separation. The utility of the prepared glyco-silicas is manifested not only for the purification of a protein mixture but also for the possibility of fluorescent labeling of the attached ConA in a single step that allows the preservation of the carbohydrate recognition domains of such a lectin. The chemical stability of the glyco-silicas and the reproducibility and recycling characteristics as affinity supports are also remarkable characteristics of the silica's viability as a universal and inexpensive scaffold in the immobilization of biomolecules.

Experimental Section

General

Unless otherwise noted, commercially available reagents and solvents were used as purchased without further purification. TLCs were performed on Merck Silica Gel 60 F_{254} aluminium sheets. Reagents used for developing plates include ceric sulfate (1% w/v) and ammonium sulfate (2.5% w/v) in 10% (v/v) aqueous sulfuric acid, iodine, ethanolic sulfuric acid (10% v/v) and by UV light when applicable. Flash column chromatography was performed on Silica Gel Merck (230–400 mesh, ASTM). Melting points were measured on a Gallenkamp melting point apparatus and are uncorrected. IR spectra were recorded on a Satellite Mattson FT-IR. ^{1}H and ^{13}C NMR spectra were recorded at room temperature on a Bruker (300–400 MHz) spectrometer. *J* values are given in Hz. FAB mass spectra were recorded on a Fisons VG Autospec-Q spectrometer, using *m*-nitrobenzyl alcohol or thioglycerol as matrix.

Synthesis of 3-Azidopropyltriethoxysilane (3)

To a solution of 3-chloropropyltriethoxysilane (**1**; 2.31 g, 9.6 mmol) and tetrabutylamonium iodide (0.020 g, 0.05 mmol) in butanone (25 mL) was added sodium azide (3.120 g, 48 mmol) and the reaction mixture heated under reflux for 50 h. After this time, filtration over celite was followed by evaporation of the solvent under vacuum. The residue was dissolved in dichloromethane (150 mL) and then washed with water (2×20 mL). The organic phase was dried ($NaSO_4$) and evaporated to give **3** (1.9 g) as a syrup that was directly used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.73$ (q, 6H, $J = 6.94$ Hz, OCH_2), 3.18 (t, 2H, $J = 7.0$ Hz, CH_2N_3), 1.62 (m, 2H, $CH_2CH_2N_3$), 1.14 (t, 9H, $J = 6.9$ Hz, OCH_2CH_3), 0.58 (m, 2H, CH_2Si); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 58.2$, 53.6, 22.5, 18.1, 7.4.

Synthesis of Propiolic Acid (3-Triethoxysilane-propyl)-amide (5)

To a solution of 3-aminopropyltriethoxysilane (**2**; 2.4 g, 10.85 mmol) and propiolic acid (0.870 g, 11.93 mmol) in dichloromethane (15 mL) was added *N*,*N*′-dicyclohexylcarbodiimide (2.460 g, 12.0 mml). The reaction mixture was kept at room temperature for 1 h and then filtered. Evaporation of the solvent was followed by coevaporation with toluene (2×50 mL) giving **5** (3.0 g) as a syrup that was directly used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.30$ (m, 1H, NH), 3.82 (q, 6H, $J = 7.0$ Hz, CH_2O), 3.28 (m, 2H, CH_2NH), 2.97 (s, 1H, C≡CH), 1.67 (m, 2H, CH_2CH_2NH), 1.22 (t, 9H, $J = 7.0$ Hz, OCH_2CH_3), 0.65 (t, 2H, $J = 8.4$ Hz, CH_2Si); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta =$ 162.5, 77.3, 73.2, 58.2, 42.0, 22.2, 17.9, 7.5.

Activation of Silica

Commercial silica (Merck, 70–230 mesh ASTM) was activated for the silanization process by heating at 120 °C under vacuum (1 mm Hg) for 24 h.

Preparation of Functionalized Silicas 4 and 6

Activated silica gel (4 g) was suspended in dried toluene (20 mL) and then the corresponding triethoxysilane derivative **3** or **6** (1 g) was added. The reaction mixture was heated under reflux for 2 h. Evaporation of the solvent up to half of the volume to remove the formed ethanol was followed by further addition of dry toluene (10 mL) and reflux for an additional hour. The reaction mixture was filtered, washed with dichloromethane (4×50 mL) and dried under vacuum (1 mm Hg) at 50 °C for 16 h.

3-{2-[2-(2-Chloroethoxy)ethoxymethyl]-3-prop-2-ynyloxy-2-prop-2-ynyloxymethylpropoxy}-propyne (10)

To a solution of **9**[35] (0.250 g, 1 mmol) and Bu_4NHSO_4 (0.678 g, 2 mmol) in 2-chloroethyl ether (5 mL)[36] was added aqueous NaOH (50%, 5 mL). The two-phase reaction mixture was vigorously stirred at room temperature for 3 h. CH_2Cl_2 (30 mL) and water (30 mL) were added , the organic phase separated and successively washed with water (2× 50 mL). The organic phase was dried (Na_2SO_4) and evaporated to give a residue that was purified by column chromatography (EtOAc-hexane, 1:3) affording **10** as a liquid; yield: 0.317 g (89%); IR (film): $\nu = 3293$, 2115, 1475, 1359, 1096 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 4.12$ (d, 6H, $J =$ 2.3 Hz, $CH_2C{\equiv}CH$), 3.80–3.55 (m. 8H, CH_2 spacer), 3.51 (s, 6H, 3 × $OCH_2C{\equiv}CH$), 3.45 (s, 2H, CH_2O spacer), 2.45 (t, 3H, $J = 2.3$ Hz, C≡CH); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta =$ 80.0, 74.1, 71.2, 71.0, 70.3, 69.7, 68.9, 58.6, 44.9, 42.8; HR-MS (FAB+): $m/z = 379.1285$, calcd. for $C_{18}H_{25}ClO_5Na$ $[M+Na]^+$: 379.1288.

Glycodendron G1-Cl (11)

A solution of the alkyne **10** (1 mmol) and the azide derivative **8** (1 mmol/alkyne function), DIPEA (3 mmol) and the cooper catalyst $[(EtO)_3P{\cdot}CuI]$[16c] (10 mmol%) in toluene (75 mL) was irradiated at 800 W and 90 °C for 20 min in a Milestone Star Microwave Labstation until TLC showed the disappearance of the starting material. The reaction mixture was evaporated and the crude material purified in a short flash column chromatography (AcOEt-MeOH, 20:1) to afford **11** as a syrup; yield: 88%; $[\alpha]_D$: +26° (*c* 1, chloroform); IR (film): $\nu = 1751$, 1431, 1371, 1226, 1138, 1091, 1047, 981 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.71$ (s, 3H,

H-5 triazole), 5.31–5.20 (m, 9H, H-2,3,4 Man), 4.82 (s, 3H, H-1 Man), 4.59 (br s, 12H, $6\times CH_2$), 4.22 (dd, 3H, $J=12.3$ and 5.0 Hz, H-6 Man), 4.05 (br d, 3H, $J=12.0$ Hz, H-6′ Man), 4.13, 3.92, 3.75–3.50 (several m, 22H), 2.14, 2.09, 2.04 and 1.99 (4 s, 36H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=170.6$, 170.0, 169.9, 169,6, 145.6, 123.8, 97.6, 71.3, 71.0, 70.4, 69.7, 69.2, 68.9, 68.9, 66.3, 65.7, 64.8, 62.2, 49.6, 45.4, 43.1, 20.9, 20.8, 20.7; MS (MALDITOF): $m/z=1630.57$, calcd. for $C_{66}H_{94}N_9O_{35}ClNa$ $[M+Na]^+$: 1630.54.

Glycodendron G1-N_3 (12)

A solution of **11** (0.746 mg, 0.46 mmol), NaN_3 (0.300 g, 4.6 mol) and Bu_4NI (0.018 g, 0.030 mmol) in DMF (8 mL) was stirred at 80 °C for 16 h. After this time ether-toluene (75 mL, 2:1) was added to the reaction mixture followed by washing with water (3×20 mL). The organic phase was dried (Na_2SO_4) and evaporated. Column chromatography (AcOEt-MeOH 20:1) of the residue gave **12** as a syrup; yield: 0.701 g (94%); $[\alpha]_D$: +21° (*c* 1, chloroform); IR (film): $\nu=2255$, 2109, 1752, 1284, 1237, 1093, 916, 731 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=7.72$ (s, 3H, H-5 triazole), 5.32–5.20 (m, 9H, H-2,3,4 Man), 4.82 (s, 3H, H-1 Man), 4.80 (m, 12H, CH_2N, CH_2O-triazole), 4.21 (dd, 3H, $J=12.7$ and 5.1 Hz, H-6 Man), 4.13 (m, 3H, CH_2O Man), 4.04 (br d, 3H, $J=12.0$ Hz, H-6′ Man), 3.93 (m, 3H, CH_2O-Man), 3.64 (t, 2H, $J=5.4$ Hz, CH_2O-spacer), 3.62–3.48 (m, 7H, H-5 Man, CH_2O spacer), 3.47 (s, 6H, pentaerythrytol), 3.43 (s, 2H, CH_2 pentaerythrytol), 3.34 (t, 2H, $J=5.0$ Hz, CH_2N_3), 2.14, 2.09, 2.04 and 1.99 (4 s, 36H, Ac); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=170.4$, 169.8, 169.7, 169.5 (COO), 145.5 (C-4 triazole), 123.6 (C-5 triazole), 97.5, 71.3, 70.9, 70.3, 69.9, 69.6, 69.1, 68.8, 68.8, 66.2, 65.7, 64,8, 62.1, 50.7, 49.5, 45.3, 20.7, 20.6; MS (MALDITOF): $m/z=1637.70$, calcd. for $C_{66}H_{94}N_{12}O_{35}Na$ $[M+Na]^+$: 1637.58.

Glycodendron G1-C≡CH (13)

A solution of **7** (0.125 g, 0.077 mmol) in MeOH (4 mL) was hydrogenated with Pd/C (7.5 mg, 6% w/w) at 2 atm for 4 h. Filtration over celite and evaporation gave a crude material that was dissolved in dry CH_2Cl_2 (5 mL). 4-Pentynoic acid (0.011 g, 0.115 mmol) and DCC (0.019 g, 0.092 mmol) were added and the reaction mixture kept at room temperature for 30 min. Filtration over celite and evaporation gave a new crude product that was purified by column chromatography (AcOEt-MeOH, 10:1) giving **13** as a syrup; yield: 0.102 g (79%); $[\alpha]_D$: +9.6° (*c* 1, chloroform); IR (film): $\nu=$ 1746, 1667, 1432, 1371, 1226, 1138, 1089, 1048 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=7.73$ (s, 3H, H-5 triazole), 6.9 (br s, 1H, NH), 5.30–5.20 (m, 9H, H-2,3,4), 4.82 (s, 3H, H-1 Man), 4.66–4.50 (m, 6H, CH_2N-triazole), 4.60 (s, 6H, CH_2-triazole), 4.22 (dd, 3H, $J=12.3$ and 5.0 Hz, H-6 Man), 4.12 (m, 3H. CH_2O-Man), 4.04 (br d, 3H, $J=12.3$ and 2.1 Hz, H-6' Man), 3.92 (m, 3H, CH_2O-Man), 3.61 (m, 3H, H-5), 3.55–3.40 (m, 16H, CH_2 spacer, $C(CH_2)_4$), 2.63–2.40 (m, 5H, $CH_2CH_2C\equiv CH$), 2.14, 2.10, 2.05 and 1.99 (4 s, 36H, Ac); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=171.3$, 170.6, 170.0, 169.9, 169.6, 145.6, 123.8, 97.6 , 83.0, 77.3, 71.0, 70.3, 69.8, 69.7, 69.1, 69.2, 68.9, 68.9, 66.3, 65.7, 64.8, 62.2, 49.6, 45.5, 39.5, 35.2, 33.0, 21.0, 20.8, 20.7, 20.7; MS (MALDITOF): $m/z=1691.71$, calcd. for $C_{71}H_{100}N_{10}O_{36}Na$ $[M+Na]^+$: 1691.62.

General Procedure for the Preparation of Glyco-Silicas 14–17

To a solution of the alkyne or azide sugar derivative **7**, **8**, **12** and **13** (0.3 mmol) in dry DMF (5 mL) was suspended the complementary azide or alkyne functionalized silica **4** or **6** (1 g) and then the copper catalyst $(EtO)_3P{\cdot}CuI$[16c] (10 mmol%, 10 mg) was added. The reaction mixture was irradiated at 800 W and 90 °C for 1 h in a Milestone Star Microwave Labstation until the IR spectra of the reaction mixture showed complete disappearance of the starting material. The reaction mixture was filtered and the resulting glyco-silica was successively washed with MeOH (2×30 mL), EDTA disodium salt solution (50 mM, 2×30 mL), water (2×30 mL), acetone (2×30 mL) and CH_2Cl_2 (2×30 mL). The glyco-silicas **14–17** were then dried under vacuum (1 mm Hg) at 50 °C for 16 h.

General Procedure for the Affinity Chromatography Assays on the Glyco-Silicas 14–17

The glyco-silicas **14–17** (0.26 g) were resuspended in 2 mL of HEPES buffer (50 mM HEPES pH 7.2, 1 mM $CaCl_2$, 1 mM $MnCl_2$) and packed into a 1 mL column. The column was equilibrated in HEPES buffer with 12 volumes of the bed (12×0.5 mL). Typically, the experiments of affinity chromatography were carried out at a flow rate of 0.3 mL/minute by means of a peristaltic pump. After loading the sample, non-immobilized protein was removed by washing the column with several volumes of 1.6 M NaCl in HEPES buffer until A_{280} was negligible. The protein retained by the column was eluted with 1 M α-D-Man*p*OMe in HEPES buffer and collected in 1 mL fractions. The purity of the sample was verified by standard SDS-PAGE[54] and silver staining.[55] The capacity of the column was estimated as the amount of ConA eluted with 1 M α-D-Man*p*OMe in HEPES buffer from the column saturated with a solution of commercial ConA in HEPES buffer (1.5 mg) and washed with 12 volumes of HEPES buffer and with 2 volumes of 0.1, 0.2, 0.4 0.8, 1.6 M NaCl in HEPES buffer.

General Procedure for Fluorescent Labeling of ConA

The fluorescent labeling of the ConA was carried out on the dimeric form of the protein immobilized in a column of the glyco-silica **16** in acetate buffer (100 mM acetate pH 5.2, 1 mM $CaCl_2$, 1 mM $MnCl_2$). Prior to the labeling the column was equilibrated with 20 volumes (10 mL) of 100 mM phosphate buffer pH 8.4 and then 2 volumes (1 mL) of 7.25 mM NBD-OCH_2CH_2OH in 100 mM phosphate buffer pH 8.4 was recirculated overnight (14 h). The unreacted excess of NBD-OCH_2CH_2OH was removed by washing with 20 volumes (10 mL) of HEPES buffer and the process was monitored by A_{280}. Finally, the labeled ConA was eluted from the column with 1 M α-D-Man*p*OMe in HEPES buffer, analyzed by SDS PAGE and visualized with a standard UV transilluminator.

Acknowledgements

We thank Dirección General de Investigación Científica y Técnica for financial support (CTQ2005–02219).

References

[1] a) K. L. Hanson, L. Filipponi, D. V. Nicolau, in: *Microarray Technology and Its Applications*, (Eds.: U. R. Müller, D. V. Nicolau), Springer, Heidelberg, **2005**, pp. 23–44; b) A. K. Mallia, P. K. Smith, *Immobilized affinity ligand techniques*, Academic Press, New York, **1992**; c) A. S. Hoffman, J. A. Hubbell, *Surface-immobilized biomolecules. Biomaterials Science* 2nd edn., Elsevier, San Diego, **2004**, pp. 225–233.

[2] R. R. Martel, M. P. Rounseville, I. W. Botros, B. E. Seligman, in: *Microarray Technology and Its Application*, (Eds.: U. R. Müller, D. V. Nicolau), Springer, Heidelberg, **2005**, pp. 3–22.

[3] a) C. M. Niemeyer, *Angew. Chem. Int. Ed.* **2001**, *40*, 4128–4158; b) C. W. C. Warren, D. J. Maxwell, X. Gao, R. E. Bailey, M. Han, S. Nie, . *Curr. Opin. Biotechnol.* **2002**, *13*, 40–46.

[4] M. Y. Han, X. H. Gao, J. Z. Su, S. Nie, *Nat. Biotechnol.* **2001**, *19*, 631–635.

[5] E. Gizeli, C. R. Lowe, *Biomolecular Sensors*, Taylor & Francis Ltd, London, **2002**.

[6] a) F. Schreiber, *J. Phys-Condens. Matter* **2004**, *16*, R881-R900; b) K. Kato, in: *Encyclopedia of Biomaterials and Biomedical Engineering,* Vol. 2 (Eds.: G. L. Bowlin, G. Wnek), Dekker, New York, **2004**, pp. 1331–1339.

[7] a) *Handbook of Affinity Chromatography*, Chromatogaphic Science Series, Vol 92, (Eds.: D. S. Hage, J. Cazes), CRC Press, New York, **2005**; b) W. Clarke, D. S. Hage, *Separ. Puri. Rev.* **2003**, *32*, 19–60.

[8] C. Doinel, M. Caron, *Bio-Sciences* **1986**, *5*, 125–133.

[9] A. Jungbauer, R. Hahn, *Curr Opin. Drug Discovery & Development* **2004**, *7*, 248–256.

[10] a) G. W. Jack, D. J. Beer, *Methods in Molecular Biology* **1996**, *59*, 187–196; b) K. Brocklehurst, A. J. Courey, S. Gul, S. H. Lin, R. L. Moritz, *Purifying Proteins for Proteomics* **2004**, 221–273.

[11] P. R. Satish, A. Surolia, *Methods for Affinity-Based Separations of Enzymes and Proteins* **2002**, 115–129.

[12] J. M. Langenhan, J. S. Thorson, *Curr. Org. Synth.* **2005**, *2*, 59–81.

[13] a) D. Wang, *Proteomics* **2003**, *3*, 2167–2175; b) M. D. Disney, P. H. Seeberger, *Drug Discov. Today: Targets* **2004**, *3*, 151–158; c) K. R. Love, P. H. Seeberger, *Angew. Chem. Inter. Ed.* **2002**, *41*, 3583–3586; d) I. Shin, S. Park, M.-R. Lee, *Chem. Eur. J.* **2005**, *11*, 2894–2901; e) M. C. Ortiz, J. M. Garcia-Fernandez, *ChemBioChem.* **2002**, *3*, 819–822.

[14] R. Huisgen, in: *1,3-Dipolar Cycloaddition Chemistry*, (Ed.: A. Padwa), Wiley, New York, **1984**, pp. 1–176.

[15] H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2001**, *40*, 2004–2021.

[16] a) C. W. Tornoe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057–3064; b) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2002**, *41*, 2596–2599; c) F. Perez-Balderas, M. Ortega-Muñoz, J. Morales-Sanfrutos, F. Hernandez-Mateo, F. G. Calvo-Flores, J. A. Calvo-Asin, J. Isac-Garcia, F. Santoyo-Gonzalez, *Org. Lett.* **2003**, *5*, 1951–1954.

[17] a) Q. Wang, S. Chittaboina, H. N. Barnhill, *Lett. Org. Chem.* **2005**, *2*, 293–301; b) H. C. Kolb, K. B. Sharpless, *Drug Discov. Today,* **2003**, *8*, 1128–1137; c) V. D. Bock, H. Hiemstra, J. H. van Maarseveen, *Eur. J. Org. Chem.* **2005**, 51–68.

[18] T. Lummerstorfer, H. Hoffmann, *J. Phys. Chem. B* **2004**, *108*, 3963–3966.

[19] S. Punna, E. Kaltgrad, M. G. Finn, *Bioconjugate Chem.* **2005**, *16*, 1536–1541.

[20] X. L. Sun, C. L. Stabler, C. S. Cazalis, E. L. Chaikof, *Bioconjugate Chem.* **2006**, *17*, 52–57.

[21] a) N. K. Devaraj, G. P. Miller, W. Ebina, B. Kakaradov, J. P. Collman, E. T. Kool, C. E. D. Chidsey, *J . Am. Chem. Soc.*, **2005**, *127*, 8600–8601; b) F. Fazio, M. C. Bryan, O. Blixt, J. C. Paulson, C. H. Wong, *J. Am. Chem. Soc.* **2002**, *124*, 14397–14402; c) M. C. Bryan, F. Fazio, H. K. Lee, C. Y. Huang, A. Chang, M. D. Best, D. A. Calarese, O. Blixt, J. C. Paulson, D. Burton, I. A. Wilson, C. H. Wong, *J. Am. Chem. Soc.* **2004**, *126*, 8640–8641.

[22] G. L. Huang, T. C. Liu, M. X. Liu, X. Y. Mei, *Anal. Biochem.* **2005**, *340*, 52–56.

[23] Y. Zhang, S. Luo, Y. Tang, L. Yu, K. Y. Hou, J. P. Cheng, X. Zeng, P. G. Wang, *Anal. Chem.* **2005**, *78*, 2001–2008.

[24] a) H. Larseriscdotter, S. Oscarsson, J. Buijs, *J. Colloid Interface Sci.* **2001**, *237*, 98–103; b) M. Van der Veen, W. Norde, M. C. Stuart, *Colloid. Surface. B.* **2004**, *35*, 33–40; c) D. Rezwan, L. P. Meier, L. J. Gauckler, *Biomaterials* **2005**, *26*, 4351–4357; d) P. M. Biesheulvel, M. Van der Veen, W. Norde, *J. Phys. Chem. B* **2005**, *109*, 4172–4180.

[25] a) A. Kondo, F. Muakami, M. Kawagoe, K. Higashitani, *Appl. Microbiol. Biotechnol.* **1993**, *39*, 726–731; b) M. Lundqvist, I. Sethson, B. H. Jonsson, *Langmuir* **2004**, *20*, 10639–10647; c) A. A. Vertegel, R. W. Siegel, J. S. Dordick, *Langmuir* **2004**, *20*, 6800–6807.

[26] H. Larsericsdotter, S. Oscarsson, J. Buijs, *J. Colloid Inteface Sci.* **2005**, *289*, 26–35.

[27] S. M. Daly, T. M. Przybycien, R. D. Tilton, *Langmuir* **2005**, *21*, 1328–1337.

[28] a) P. M. Price, J. H. Clark, D. J. Macquarrie, *Dalton Trans.* **2000**, 101–110; b) P. K. Jal, S. Patel, B. K. Mishra, *Talanta* **2004**, *62*, 1005–1028.

[29] H. Lakhiari, J. Jozefonvicz, D. Muller, *J. Chromatogr. B* **1998**, *76*, 33–41.

[30] G. Rodriguez-Lopez, M. D. Marcos, R. Martinez-Manez, F. Sancenon, J. Soto, L. A. Villaescusa, D. Beltran, P. Amoros, *Chem. Commun.* **2004**, 2198–2199.

[31] a) A. Heeboll-Nielsen, M. Dalkiaer, J. J. Hubbuch, O. R. T. Thomas, *Biotechnol. Bioeng.* **2004**, *87*, 311–323; b) W. C. Lee, C. C. Hsiao, R. C. Ruaan, *J. Chem. Technol. Biotechnol.* **1995**, *64*, 66–72.

[32] A. Karl, W. Buder, Eur. Patent 50768 A2 19820505, **1982**; *Chem. Abst.* **1982**, *97*, 163246.

[33] R. Roy, S. K. Das, F. Santoyo-Gonzalez, F. Hernandez-Mateo, T. K. Dam, C. F. Brewer, *Chem.Eur. J.* **2000**, *6*, 1757–1762.
[34] A. Y. Chernyak, G. V. M. Sharma, L. O. Kononov, P. R. Krishna, A. B. Levinsky, N. K. Kochetkov, A. V. R. Rao, *Carbohydr. Res.* **1992**, *223*, 303–309.
[35] F. G. Calvo-Flores, J. Isac-Garcia, F. Hernandez-Mateo, F. Perez-Balderes, J. A. Calvo-Asín, E. Sanchez-Vaquero, F. Santoyo-Gonzalez, *Org. Lett.* **2000**, *2*, 2499–2502.
[36] a) P. Bako, L. Toke, *J. Inclusion Phenom. Mol. Recogn.* **1995**, *23*, 195–201; b) P. Bako, T. Bako, K. Bisztray, A. Szollosy, K. Nagy, L. Toke, *J. Inclusion. Phenom.* **2001**, *39*, 247–251.
[37] L. E. Samuelson, K. B. Sebby, E. D. Walter, D. J. Singel, M. J. Cloninger, *Org. Biomol. Chem.* **2004**, *2*, 3075–3079.
[38] E. Gasteiger, C. Hoogland, A. Gattiker, S. Duvaud, M. R. Wilkins, R. D. Apple, A. Bairoch, in: *The Proteomics Protocol Handbook*, (Ed.: J. M. Walker), Humana Press Inc, Totowa, N. J., **2005**, pp. 571–607.
[39] J. J. Valle-Delgado, J. A. Molina-Bolivar, F. Galisteo-Gonzalez, M. J. Galvez-Ruiz, A. Feiler, R. W. Rutland, *J. Phys. Condens. Matter* **2004**, *16*, S2383-S2392.
[40] a) W. Norde, J. P. Favier, *Colloid. Surface* **1992**, *64*, 87–93; b) H. Larsericsdotter, S. Oscarsson, J. Buijs, *J. Colloid Interface Sci.* **2005**, *289*, 26–35.
[41] S. Koutsopoulos, J. van der Oost, W. Norde, *Biophys. J.* **2004**, *88*, 467–474.
[42] T. Arai, W. Norde, *Colloid. Surface* **1990**, *51*, 1–15.
[43] R. Loris, T. Hamelryck, J. Bouckaert, L. Wyns, *Biochim. Biophys. Acta* **1998**, *1383*, 9–36.
[44] A. Salahuddin, R. Begum, B. K. Averill, *Biochem J.* **1984**, *22,* 639–642.
[45] W. C. Lee, K. H. Lee, *Anal. Biochem.* **2004**, *324*, 1–10.
[46] V. Horejsi, J. Kocoarek, *Biochim. Biophys. Acta* **1978**, *53,*: 299–315.
[47] a) M. Caron, A. P. Seve, D. Badier, R. Joubert-Caron, *J. Chromatogr. B* **1998**, *715* 153–161; b) M. Caron, R. Joubert-Caron, J. R. Cartier, A. Chadli, D. Bladier, *J. Chromatogr.* **1993**, *646*, 327–333.
[48] A. Waggoner, *Curr. Opin. Chem. Biol.* **2006**, *1,*:62–66.
[49] S. Uchiyama, T. Santa, N. Okiyama, T. Fukushima, K. Imai, *Biomed. Chromatogr.* **2001**, *15*, 295–318.
[50] L. Johnson, S. Lagerkvist, P. Lindroth, M. Ahnoff, K. Martinsson, *Anal. Chem.* **1982**, *54*, 939–942.
[51] M. J. Waner, M. Gilchrist, M. Schindler, M. Dantus, *J. Phys. Chem. B* **1998**, *102*, 1649–1657.
[52] P. B. Ghosh, M. W. Whitehouse, *Biochem. J.* **1968**, *108*, 155–156.
[53] D. Birkett, N. C. Price, G. K. Radda, A. G. Salmon, *FEBS Lett.* **1970**, *6*, 346–348.
[54] U. K. Laemmli, *Nature* **1970**, *227*, 680–685.
[55] C. R. Merril, D. Goldman, M. L. Van Keuer, *Meth. Enzymol.* **1984**, *104*, 441–447.

