Vinyl sulfone: a versatile function for simple bioconjugation and immobilization†

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The easy functionalization of tags and solid supports with the vinyl sulfone function is a valuable tool in *omic* sciences that allows their coupling with the amine and thiol groups present in the proteogenic residues of proteins, in mild and green conditions compatible with their biological function.

Introduction

The covalent coupling of two biomolecules to each other (bioconjugation) or to a solid support (immobilization) is one of the cornerstones of *omic* sciences.[1] Thus, labeling with biophysical probes and biotinylation of proteins are standard techniques nowadays, while tethering of biomolecules to solid supports is exploited in the fabrication of arrays and in the immobilization of enzymes. For proteins, the straightforward and probably most widely used methodologies take advantage of the reactivity of the naturally occurring functional groups present in proteogenic amino acids towards labeling reagents or supports, commonly by means of a nucleophile-to-electrophile attack. Lysine is by far the most targeted residue because of its predominant presence (the 11th most frequent residue),[2] the reactivity of the ε-amine group of its side chain, its minor relevance from a biological point of view, and its accessibility at the surface of those biomolecules. Amine-reactive labels are usually acylating agents such as sulfonyl chlorides, isothiocyanates and succinimidyl esters. However, they are not exempt from drawbacks. Sulfonyl chlorides are highly reactive but also unstable in water,[3] especially at the high pH required for the reaction with aliphatic amines, and they can also react with phenols (tyrosine), aliphatic alcohols (serine, threonine), thiols (cysteine) and imidazoles (histidine). Isothiocyanates are stable in water, although their reactivity is only moderate, and the degradation of the resulting thiourea has been reported.[4] Succinimidyl esters are the best suited amine reagent for derivatization, and the less reactive but more soluble sulfosuccinimidyl esters have been used to overcome their poor water solubility.[5] For these reasons, the chemical modification of complex biomolecules, such as proteins, by simple procedures under physiological conditions remains a challenge.

In this context, the Michael-type addition of vinyl sulfones[6] is an attractive methodology for protein conjugation.[7] Up to the present, vinyl sulfone chemistry has been demonstrated to be suitable for the selective modification of cysteine residues under mild conditions, the second least abundant amino acid in proteins with a frequency of 1.36%.[2] The water stability of the vinyl sulfone function, the lack of by-products, the almost quantitative yields of the reaction with thiols and the stability of the thioether linkage formed are appealing characteristics of this reaction that in practice, however, has been used almost exclusively for PEGylation of proteins using end-functionalized vinyl-sulfone PEG derivatives.[8] In contrast with the facile functionalization of cysteine by reaction with vinyl sulfones, the reaction of this function with lysine residues has been reported[8a,9] to be slow and incomplete, occurring only at high pH values (pH >9.3), conditions not always compatible with the biological function of proteins.

Taking into account our previous results on the good reactivity of primary amines with vinyl sulfone sugar derivatives,[10] we decided to explore the feasibility of using the vinyl sulfone function in bioconjugation under mild conditions. We report here on the capabilities of the vinyl sulfone function for the covalent coupling of proteins to detection labels, other biomolecules and solid supports by exploiting the Michael-type addition of this function to the amine-containing residues, in conditions that preserve the functionality and biological integrity of those biological macromolecules.

Results and discussions

Synthesis of vinyl sulfone-derivatized reagents

To study the value of the vinyl sulfones as a viable new tool in the arsenal of bioconjugation strategies, we introduced the vinyl sulfone function to a set of commonly used synthetic tags with a biotechnological interest in bioconjugation such as rhodamine B and dansyl (fluorescent tags), biotin (affinity tag), and ferrocene (electrochemical tag),[11] as well as on glucose, chosen as an example of a simple biomolecule (Scheme 1).

The vinyl sulfone-derivatization of the fluorescent tags and biotin was carried out by reaction of a scaffold bearing both a vinyl sulfone group and an amine group with commercial rhodamine B and biotin, previously transformed into the corresponding acid chloride derivatives, and dansyl chloride. The synthesis of such an amine–vinyl sulfone scaffold was approached by reaction of a bis-vinyl sulfone with a primary amine. To avoid the reported intramolecular process in the reaction of divinyl sulfone with

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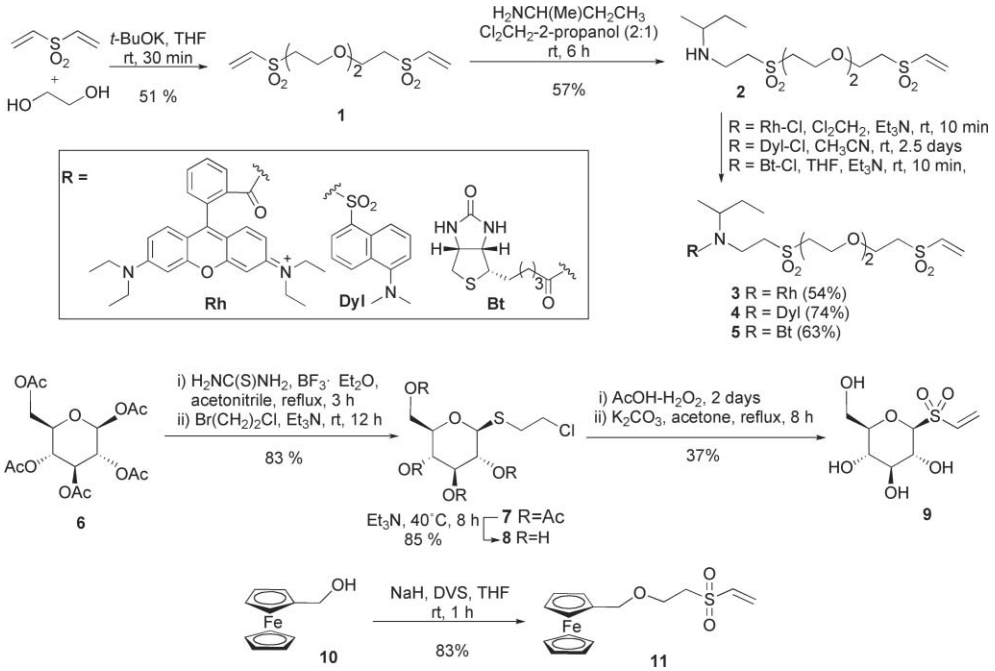


Scheme 1 Synthesis of vinyl sulfone-functionalized tags and sugars.

primary amines leading to 1,4-thiazane-1,1-dioxide derivatives,[12] a longer bis-vinyl sulfone (**1**) was synthesized by reaction of divinyl sulfone with ethylene glycol. However, the reaction of **1** with ethanolamine or propylamine did not lead to the desired monoaddition products and, to overcome this difficulty, the use of a steric hindrance amine was envisioned as a feasible alternative. By this route, the desired amine–vinyl sulfone scaffold **2** was obtained in 57% yield by reaction of **1** with *sec*-butylamine in Cl_2CH_2 : isopropanol. Treatment of the amine–vinyl sulfone **2** with rhodamine B acid chloride and dansyl chloride gave the fluorescent labels **3** and **4**, in 54% and 74% yield, respectively. The reactions were carried out in anhydrous Cl_2CH_2 and anhydrous acetonitrile, respectively, because the former overcame the solubility problems of rhodamine B acid chloride, and the latter led to shorter reaction times and higher yields. The same procedure was used for the coupling of biotin acid chloride in anhydrous THF in the presence of Et_3N, leading to the biotinylation reagent **5** in 63% yield.

On the other hand, the vinyl sulfone-functionalized glucose derivative **9** was obtained by oxidation and elimination of the 2-chloroethyl-1-thio-β-D-glucopyranoside **8**, which was easily accessible from per-*O*-acetylated-β-D-glucose by successive treatment with thiourea and 1-bromo-2-chloroethane, and subsequent de-*O*-acetylation. Finally, vinyl sulfone ferrocene **11** was easily obtained by reaction of methanol ferrocene **10** with divinyl sulfone (Scheme 1).

Bioconjugation of proteins by vinyl sulfone-functionalized reagents

The vinyl sulfone-derivatization described above yields activated electrophilic olefin reagents that may react with nucleophiles by a Michael-type addition. In mild conditions, amine and thiol groups may deprotonate and act as Michael donors, but for the particular case of proteins, their accessibility and pK_a values are affected by the surrounding residues. The degree to which a protein is labeled is also dependent on the conjugation process, and labeling reactions are influenced by both the molar ratio of the reactants and the activity of the labeling reagent. Considering these facts, and in order to put to the test our hypothesis about the bioconjugation capabilities of the synthesized vinyl sulfone reagents, a pool of four commercial proteins (avidin, Concanavalin A (ConA), lysozyme and BSA), comprising both acidic and alkaline isoelectric points, monomeric and oligomeric structures, different numbers of potential Michael donors and the presence/absence of free thiol groups, and post-translational modifications were selected (Table 1).

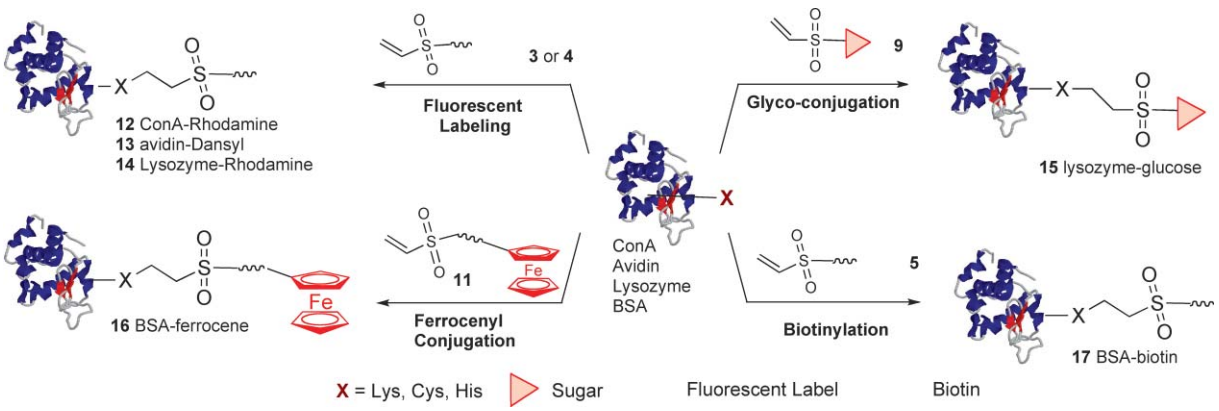
The evaluation of the vinyl sulfone-derivatization of tags to label proteins in mild conditions was carried out in the first instance on fluorescent labels **3** and **4** that were conjugated with ConA and avidin, respectively, to facilitate the monitoring of the reaction (Scheme 2). The conjugation process was carried out at pH 8, 37 °C, and with different stoichiometries and reaction times (3, 8 and 24 h). After analysis of conjugates **12** and **13** by SDS-PAGE, the results showed that ConA is labeled very efficiently within 3 h, with no appreciable differences detected between 8 and 24 h of reaction, while avidin is poorly labeled within 3 h and longer reaction times yield more intense fluorescence (Fig. 1).

In spite of the number of potential nucleophiles (20 for ConA *versus* 10 for avidin), the molar excess of the labeling reagent was depleted by the latter but not by the former. A closer analysis reveals that higher stoichiometries do not yield more intense fluorescence, but they provoke the precipitation of ConA (ESI, Fig. S1†), probably due to the hydrophobicity associated with the modification of the hydrophilic nature of the lysine residues, and in the case of avidin, even less intense fluorescence, whose over-labeling causes quenching (ESI, Fig. S2†). These results demonstrate that the protein itself influences the extent of the coupling, and that the labeling of proteins with vinyl

Table 1 Main features of the pool of model proteins

Entry	Protein	Quaternary structure	Molecular weight/kDa[a]	Isoelectric point[a]	Post-translational modifications	Nucleophilic residues (per monomer)			
						Lys	His	Cys (free)	S–S bonds
1	ConA	Tetramer	4 × 26.6	5.3	None	12	6	None	None
2	Avidin	Tetramer	4 × 17	9.5	Glycosylation	9	1	None	1
3	Lysozyme	Monomer	14.3	11	None	6	1	None	4
4	BSA	Monomer	66.5	5.6	None	59	17	1	17

[a] Estimated from the sequence, except for lysozyme whose isoelectric point was taken from Alderton *et al.*[13]



Scheme 2 Applications of vinyl sulfone-functionalized tags and sugars in bioconjugation of proteins.

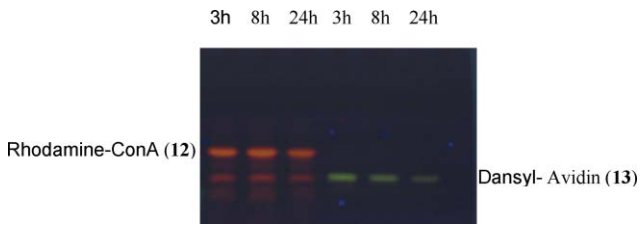


Fig. 1 SDS-PAGE of the reaction of ConA with **3** (red) and avidin with **4** (green) for 3, 8 and 24 h (from left to right) (conjugates **12** and **13**, respectively).

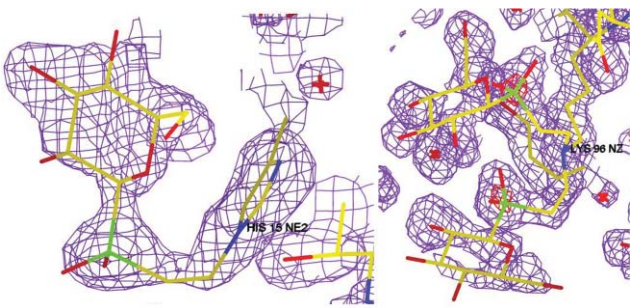


Fig. 2 Identification of residues present in the HEW lysozyme that react with vinyl sulfone glucose **5**. 2mFo-DFc maps contoured at 1.2 σ (blue) or 5 σ (red) showing the electron density that defines the sugars bonded to Lys96 (right) and His15 (left).

sulfone-derivatized reagents is feasible regardless of the isoelectric point, number of potential nucleophiles or glycosylation.[14]

Having demonstrated the conjugation of vinyl sulfone-functionalized reagents with proteins lacking free Cys under mild conditions, we focused on the identification of the amino acid residues that react with the vinyl sulfone function. For this purpose, HEW lysozyme was glycosylated by reaction with glucopyranosyl vinyl sulfone **9** in phosphate buffer pH 7.7 at room temperature. The resulting conjugate **15** (Scheme 2) was crystallized by hanging drop, and the structure solved at 1.6 Å by X-ray diffraction (PDB entry 2B5Z). The electron density map clearly revealed that both Hys and Lys residues act as Michael donors in the reaction with **9**.[15] These results prove that, contrary to the generally accepted view, Lys residues not only react with a vinyl sulfone-containing reagent at a pH compatible with the biological nature of the sample, but that they may also yield a double addition process (Fig. 2).

Influence of the reaction conditions on the bioconjugation of vinyl sulfone-functionalized reagents

We next addressed the influence of the reaction conditions on the coupling of vinyl sulfone-functionalized reagents to model proteins. First, HEW lysozyme was labeled with vinyl sulfone rhodamine B **3** at pH ranging from 5 to 8, and both at room temperature and 30 °C (conjugate **14**, Scheme 2). The resulting labeled proteins were analyzed by MALDI-TOF (Fig. 3), which showed that the reaction takes place even in acidic media, and that slight variations of pH or temperature exert a clear direct effect on the number of labels (**3**) coupled to the HEW lysozyme, *i.e.* the number of fluorescent tags increases with pH or temperature. Second, it was demonstrated that the stoichiometry, in terms of the vinyl sulfone groups : protein reactive groups (*i.e.* Lys, His and Cys residues) ratio, also plays a role. Thus, the extent of the

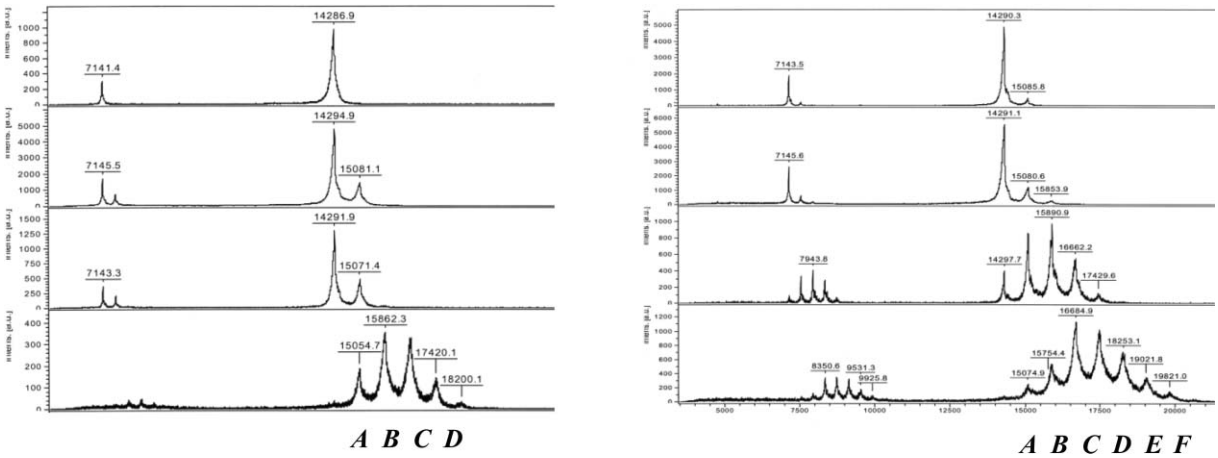


Fig. 3 MALDI-TOF spectra of the HEW lysozyme labeled with vinyl sulfone rhodamine B **3** at rt (left) and 30 °C (right), and pH 5, 6, 7 and 8 (from top to bottom). Molecular weight of lysozyme is 14 290 and that is increased by 796.4 Da per molecule of rhodamine B **3** coupled: ***A*** HEW + 1Rh; ***B*** HEW + 2Rh; ***C*** HEW + 3Rh; ***D*** HEW + 4Rh; ***E*** HEW + 5Rh; ***F*** HEW + 6Rh.

Table 2 Influence of the stoichiometry on the labeling of BSA with vinyl sulfone ferrocene **11**

BSA : **11**/mol[a]	BSA reactive groups : **11**	Fe per BSA/mol
1 : 10	1 : 0.13	9.28
1 : 50	1 : 0.65	25.03
1 : 100	1 : 1.30	28.75

[a] Estimated from the determination of Fe present in the sample resulting from the coupling.[16]

labeling of BSA with the vinyl sulfone ferrocene electrochemical tag **11**, at pH 8.5 and room temperature, that yielded conjugated **16** (Scheme 2), showed that at an **11** : BSA ratio as unfavourable as 0.13 : 1 (*i.e.* 10 mols of **11** per mol of BSA) yielded between 9 and 10 labels per molecule of BSA. As the **11** : BSA ratio increased, the extent of labeling also increased to reach a maximum that corresponded to less than 30 labels (see Table 2). A closer analysis of these results based on the existence in BSA of a single free Cys and 77 reactive groups further demonstrates the good reactivity of amine groups present in proteins towards vinyl sulfones, and the existence of reactive groups that do not react.

Functionality of the vinyl sulfone-labeled model proteins

Although fluorescent labeling is an important application, macromolecule labeling is also aimed at promoting/detecting the interaction between the labeled molecule and others. In this context, the avidin–biotin technology[17] plays a central role, and the introduction of the avidin–biotin complex into a given system is a widely used approach that serves to mediate between a recognition system (*e.g.* antibody–antigen or lectin–carbohydrate) and a reporter group (*e.g.* enzymatic activity or fluorescence). To explore this range of applications with the new vinyl sulfone tags, BSA was labeled with vinyl sulfone-derivatized biotin **5** and the resulting conjugate **17** combined with the dansyl-labeled avidin conjugate **13** reported above (Scheme 2).[14] The functionality of both the biotinylated BSA and the fluorescently labeled avidin was analyzed by SDS-PAGE, since the avidin–biotin interaction is one of the strongest non-covalent biological interactions ($K_a = 10^{15}$ M^{-1}), and is considered as essentially non-reversible and unaffected by denaturing agents. Thus, after denaturation of the sample in mild conditions, the SDS-PAGE showed a fluorescent signal at a high molecular weight that corresponded to BSA–biotin–avidin–dansyl complexes (Fig. 4, left). In order to further assess the identity of these complexes, a new electrophoresis was carried out with samples being denatured in standard conditions (Fig. 4, right). The gel demonstrated that the fluorescence at high molecular weights did not correspond to BSA since its molecular weight is higher, and it disappeared when more stringent denaturation conditions were employed. These results demonstrate that the fluorescent high molecular weight band consists of BSA–biotin–avidin–dansyl complexes, and that the coupling of vinyl sulfone-derivatized biotin and dansyl yield fully functional species.

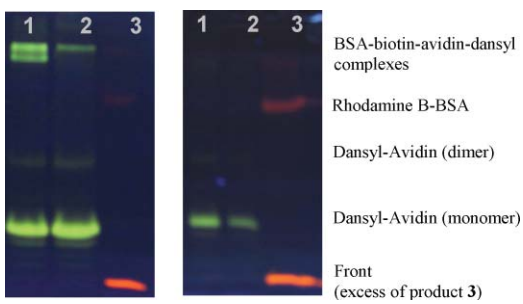


Fig. 4 SDS-PAGE, in mild (left) and standard (right) denaturing conditions, of the complex BSA–biotin–avidin–dansyl at BSA–biotin (**17**) : avidin–danysl (**13**) stoichiometries of 4 : 4 (lane 1) and 4 : 1 (lane 2). Lane 3 is BSA pre-stained with **3**.

Considering that avidin is a tetramer, a 4 : 4 stoichiometry of dansyl–avidin : biotin–BSA should yield a full occupancy of the four biotin binding sites, and a concomitant more intense fluorescence than that for a 4 : 1 stoichiometry. As expected, this higher intensity was observed. However, in both cases, single species were detected by Coomassie because either they did not interact, or they did not withstand the mild denaturing conditions (ESI, Fig. S3†).

Vinyl sulfone-derivatized rhodamine B as a pre-stain reagent for electrophoresis

During the electrophoresis performed in the labeling assays described above, it was noticed that the bright purple color of the vinyl sulfone-derivatized rhodamine B **3** was transferred to the labeled protein and made it visible to the naked eye. This feature suggests a potential application in SDS-PAGE as a pre-stain dye that, besides yielding fluorescence for sensitive detection/quantification, allows monitoring of the protein migration during the electrophoretic separation. In fact, the use of Remazol dyes, which at alkaline pH are converted to their vinyl sulfone derivatives, was proposed as a pre-stain reagent for visualization of proteins in SDS–polyacrylamide gels.[18]

Rhodamine–vinyl sulfone (**3**) was put to the test, bearing in mind that a pre-stain dye may be attractive if (i) the protocol of pre-staining is straightforward and general, (ii) the detection limit is good enough, (iii) the migration pattern remains unaltered, and (iv) the pre-staining is compatible with conventional post-electrophoresis stains. The study was carried out on the model monomeric proteins BSA and lysozyme (Table 1). BSA, with an isoelectric point of 5.6 and 77 potential nucleophilic residues, among them 1 free Cys, is a good model for an acidic protein, while lysozyme, with an alkaline isoelectric point of 11 and only 7 potential nucleophilic residues, represents an extreme case of a protein with low reactivity expected towards vinyl sulfone. The results led to the conclusion that a procedure as simple as incubation of the sample with the vinyl sulfone-derivatized rhodamine B **3** at 100 °C for 5 min, in HEPES pH 8.8, yielded the detection of roughly 1 μg by the purple color (Fig. 5) and 125 ng by fluorescence in a conventional transilluminator (Fig. 6). The subsequent staining of the sample revealed that the pre-staining is fully compatible with a post-electrophoresis silver stain, and electrophoretic mobility is not altered (Fig. 6). Improvement in the sensitivity of the detection may be achieved by exciting the sample at a more suitable wavelength (ESI, Fig. S4†) and recording on a CCD camera.[14]

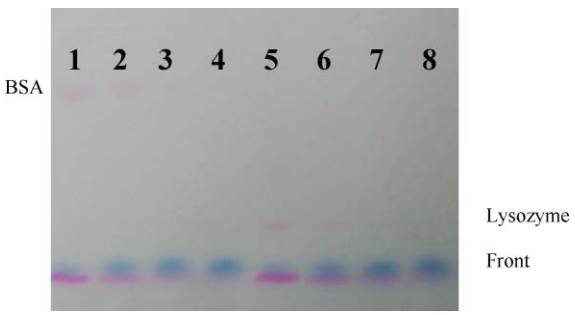


Fig. 5 Sensitivity of pre-staining BSA (lanes 1 to 4) and lysozyme (lanes 5 to 8) with vinyl sulfone rhodamine (**3**). The amount of pre-stained protein loaded was 5 μg (lanes 1 and 5), 2.5 μg (lanes 2 and 6), 1.25 μg (lanes 3 and 7) and 0.625 μg (lanes 4 and 8).

Further experiments demonstrated that shorter incubation times were insufficient, and that longer times or a larger molar excess of vinyl sulfone-derivatized rhodamine B **3** yielded stronger fluorescence at the cost of retarding the mobility of the proteins, a common drawback described for other lysine-modifying agents.[19] At this point, it is important to remark on the importance of decoupling pre-staining from denaturation, since the latter is carried out in a mixture containing amine and thiol groups that react with the vinyl sulfone groups. However, the penalty of the decoupling is an affordable short incubation of 10 min at no risk, since pre-staining with vinyl sulfone-derivatized rhodamine B **3** does not interfere with a conventional post-electrophoresis silver stain (Fig. 6).

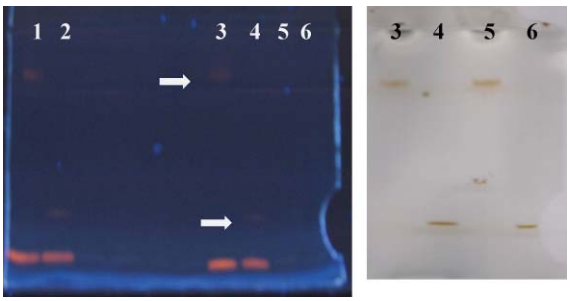


Fig. 6 SDS-PAGE of pre-stained samples. Fluorescence (left) of BSA (lanes 1 and 3) and lysozyme (lanes 2 and 4) pre-stained with vinyl sulfone rhodamine **3**, and post-electrophoresis silver staining (right). The amount of protein per lane was 250 ng (lanes 1 and 2) and 125 ng (lanes 3 to 6). Control non-pre-stained samples were in lanes 5 and 6.

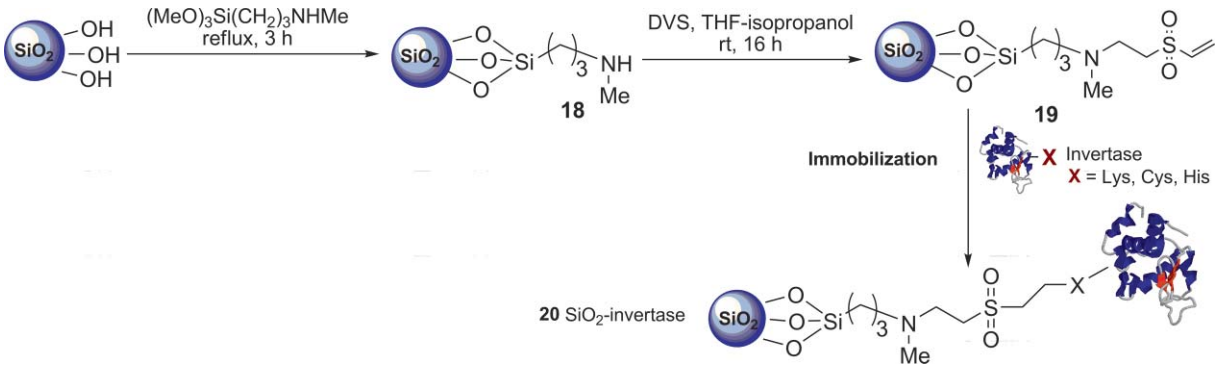
Immobilization of proteins by vinyl sulfone silica

Another explored area of application of the vinyl sulfone group's reactivity towards biomolecules was immobilization onto solid supports. The preservation of the biological functionality of the biomolecules once immobilized is a requisite of primary importance. In this context, we chose silica as a solid support based on the appealing characteristics of silica-based hybrid materials.[20] Our approach was based on the vinyl sulfone-functionalization of silica that was easily performed by silanization of activated commercial silica with [3-(methylamine)propyl]-trimethoxysilane and subsequent reaction with divinyl sulfone (Scheme 3).[21]

The capability of the resulting vinyl sulfone silica **19** to immobilize biomolecules while preserving their functionality was then evaluated using invertase (Scheme 3). Invertase is a model enzyme that catalyzes the hydrolysis of sucrose to fructose and glucose, a process of interest to the food industry. The immobilization on vinyl sulfone silica **19** was carried out in batch, at both room temperature and 4 °C, by simple addition of the functionalized silica to a solution of the enzyme in a standard phosphate buffer at pH 7.5. The results show that the reaction takes place even at 4 °C, although with lower yield, as expected from the influence of the temperature on the reaction, but the enzymatic activity is preserved, as the specific activity of the enzyme immobilized at 4 °C to hydrolyze sucrose is about 2.5 times larger (Table 3).[21]

Table 3 Immobilization of invertase onto vinyl sulfone silica **19** and functionality of the immobilized enzyme **20** to hydrolyze sucrose

Temperature	Immobilized invertase/mg invertase per gram **19**	Specific activity of immobilized invertase/arbitrary units per mg immobilized invertase
rt	100.8	1.40
4 °C	50.2	3.44



Scheme 3 Synthesis of vinyl sulfone silica **19** and immobilization of proteins.

Conclusions

In conclusion, the Michael-type addition of amine and thiol groups naturally occuring in biomolecules to vinyl sulfone is a methodology wide in scope. The novelty of the methodology presented herein relies on the ability of a protein to react with vinyl sulfone-functionalized reagents *via* the amine groups present in the side chain of their Lys and His residues, in mild conditions that preserve the biological functionality of proteins. The approach is a general strategy for bioconjugation and immobilization of proteins. The results described herein demonstrate the feasibility of the vinyl sulfone-derivatization to tackle the fluorescent labeling of proteins and their biotinylation, two important tools in *omic* sciences, and also the capability of vinyl sulfone silica to act as a universal support to immobilize proteins while preserving their functionality. Moreover, the vinyl sulfone-derivatized rhodamine B is a suitable reagent for the routine labeling of proteins prior to electrophoretic separation, the pre-staining being compatible with a later standard silver or Coomassie Brilliant Blue staining. Although the protein itself influences the extent of the coupling, the reactions take place regardless of their isoelectric point, number of potential nucleophiles, or the presence of post-translational modifications or free Cys residues, and the reaction is simple (it involves the simple combination of both species), efficient (it takes place even at 4 °C) and "green" as it requires benign solvent (*i.e.* aqueous media), leading to stable linkages with an absence of by-products. In addition, it can be anticipated that in the context of life sciences, the scope of the vinyl sulfone function will also involve its use as a practical convergent approach for protein post-translational modifications that will expand the actual repertory of methodologies, as demonstrated with the glycosylation of lysozyme

Experimental

General experimental procedures

TLC was performed on Merck Silica Gel 60 F254 aluminium sheets. Reagents used for developing plates include potassium permanganate (1% w/v), ninhydrin (0.3% w/v) in ethanol and 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. Optical rotations were recorded on a Perkin-Elmer 141 polarimeter at room temperature. IR spectra were recorded on a Satellite Mattson FTIR. ^{1}H and ^{13}C NMR spectra were recorded at room temperature on a Varian Direct Drive (300, 400 and 500 MHz) spectrometer. Chemical shifts are given in ppm and referenced to internal $CDCl_3$. *J* values are given in Hz. FAB mass spectra were recorded on a Fissons VG Autospec-Q spectrometer, using *m*-nitrobenzyl alcohol or thioglycerol as matrix. MALDI-TOF and NALDI-TOF mass spectra were recorded on an Autoflex Brucker spectrometer using HCCA and NaI, respectively, as matrix. Divinylsulfone, rhodamine B, dansyl chloride, biotin, ferrocene methanol, lysozyme, ConA, avidin, BSA, *Saccharomyces cerevisiae*, and *sec*-butylamine were purchased from commercial suppliers.

Synthesis of vinyl sulfone tags and reagents

Synthesis of bis-vinyl sulfone 1. *t*-BuOK (119 mg, 1.1 mmol) was added to a solution of divinyl sulfone (DVS) (1.6 mL, 16 mmol) and ethylene glycol (330 mg, 5.3 mmol) in THF (100 mL). The reaction mixture was stirred at rt for 30 min. Removal of the solvent under reduced pressure yielded a crude that was purified by column chromatography (AcOEt–hexane 2 : 1 → 3 : 1) giving **1** (805 mg, 51%) as a syrup. ν_{max}(film)/cm^{-1}: 1608, 1472, 1382, 1310, and 1121; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 6.76 (dd, 2 H, *J* 16.6 and 9.8 Hz), 6.41 (d, 2H, *J* 16.6 Hz), 6.10 (d, 2 H, *J* 9.8 Hz), 3.90 (t, 4 H, *J* 5.8 Hz), 3.63 (s, 4 H), 3.25 (t, 4 H, *J* 5.6 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 137.9, 129.1, 70.3, 64.7, 55.0. HRMS (*m*/*z*) (FAB+) calcd. for $C_{10}H_{18}O_6S_2Na$ [M + Na]$^+$: 321.0442; found: 321.0442.

Synthesis of amino vinyl sulfone 2. To a solution of **1** (1.0 g, 3.3 mmol) in Cl_2CH_2–2-propanol (2 : 1, 45 mL) was added *sec*-butylamine (227 µL, 2.2 mmol). The reaction mixture was kept at rt for 6 h. Evaporation of the solvent yielded a crude that was purified by column chromatography (AcOEt → AcOEt–MeOH 10 : 1) giving **2** (472 mg, 57%) as a syrup. ν_{max}(film)/cm^{-1}: 2961, 2922, 2872, 1604, 1459, 1378, 1311, 1287, and 1121; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 6.71 (dd, 1 H, *J* 16.6 and 10 Hz), 6.34 (d, 1 H, *J* 16.6 Hz), 6.06 (d, 1 H, *J* 10 Hz), 3.84 (m, 4 H), 3.58 (s, 4 H), 3.29 (t, 2 H, *J* 5.3 Hz), 3.21 (m, 4 H), 3.06 (m, 2 H), 2.53 (m, 1 H), 2.00 (br s, 1 H), 1.42 (m, 1 H), 1.27 (m, 1 H), 0.98 (d, 3 H, *J* 6.3 Hz), 0.83 (t, 3 H, *J* 7.4 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 137.9, 129.1, 70.4, 70.3, 64.8, 64.6, 55.3, 54.9, 54.6, 54.4, 40.3, 29.4, 19.6, 10.1. HRMS (MALDI-TOF) calcd. for $C_{14}H_{29}O_6NS_2Na$ [M + Na]$^+$: 394.1334; found: 394.1334.

Synthesis of vinyl sulfone rhodamine B 3. A solution of rhodamine B (340 mg, 0.71 mmol) in Cl_2SO (7 mL) was kept at rt overnight. The reaction mixture was evaporated under vacuum and co-evaporated with anhydrous toluene (3 × 15 mL) to give rhodamine B acid chloride. The crude acid chloride was dissolved in anhydrous CH_2Cl_2 (10 mL) and added dropwise to a solution of **2** (240 mg, 0.65 mmol) and Et_3N (184 μL, 1.29 mmol) in anhydrous CH_2Cl_2 (15 mL). The reaction mixture was kept at rt for 10 min. Evaporation of the solvent yielded a crude that was purified by column chromatography (CH_2Cl_2–MeOH 30 : 1 → 10 : 1) giving **3** as a foam solid (278 mg, 54%). ν_{max}(film)/cm^{-1}: 1640, 1589, 1466, 1413, 1339, 1275, 1180, 1128; ^{1}H-NMR (Cl_3CD, 500 MHz): δ 7.71-7.55 (several m, 3 H), 7.39-7.23 (several m, 3 H), 7.03 (d, 1 H, *J* 9.4 Hz), 6.89 (d, 1 H, *J* 10 Hz), 6.82 (br s, 2 H), 6.75 (dd, 1 H, *J* 16.6 and 9.9 Hz), 6.33 (d, 1 H, *J* 16.6 Hz), 6.07 (d, 1 H, *J* 9.9 Hz), 3.87-3.57 (several m, 21 H), 3.23 (t, 2 H, *J* 5.7 Hz), 3.13 (t, 2 H, *J* 5.3 Hz), 1.43-1.26 (several m, 14 H), 0.89-0.67 (several m, 6 H); ^{13}C-NMR (Cl_3CD, 125 MHz): δ 169.5, 157.8, 157.6, 155.7, 155.0, 137.9, 136.1, 132.6, 130.5, 130.4, 129.8, 129.5, 128.9, 128.2, 114.6, 113.7, 113.6, 113.5, 96.6, 96.5, 70.3, 70.2, 67.1, 64.5, 64.2, 56.7, 54.8, 53.7, 52.5, 46.3, 46.2, 34.6, 18.4,13.7, 12.7, 11.2; HRMS (*m*/*z*) (MALDI-TOF) calcd. for $C_{42}H_{58}N_3O_8S_2$ [M]$^+$: 796.366; found: 796.366.

Synthesis of vinyl sulfone dansyl 4. To a solution of dansyl chloride (130 mg, 0.48 mmol) in anhydrous acetonitrile (15 mL) were added **2** (150 mg, 0.40 mmol) and Et_3N (115 μL, 0.8 mmol). The reaction mixture was kept at rt for 2.5 d. Evaporation of the solvent yielded a crude that was purified by column chromatography (AcOEt–hexanes 1 : 1 → 3 : 1) giving **4** as a syrup (182 mg, 74%). ν_{max}(film)/cm^{-1}: 2929, 2871, 1569, 1456, 1387, 1311, and 1132 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 8.55 (d, 1 H, *J* 8.6 Hz), 8.28 (d, 1 H, *J* 8.6 Hz), 8.24 (d, 1 H, *J* 7.4 Hz), 7.53 (m, 2 H), 7.17 (d, 1 H, *J* 7.6 Hz), 6.73 (dd, 1 H, *J* 16.6 and 10 Hz), 6.37 (d, 1 H, *J* 16.6 Hz), 6.05 (d, 1 H, *J* 10 Hz), 3.91 (m, 4 H), 3.80-3.46 (several m, 9H), 3.27 (t, 2 H, *J* 5.9 Hz), 3.22 (m, 2 H), 2.87 (s, 6 H), 1.38 (m, 2 H), 0.98 (d, 3 H, *J* 6.6 Hz), 0.65 (t, 3 H, *J* 7.3 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 138.0, 134.9, 130.9, 130.7, 130.3, 130.2, 129.1, 128.5, 123.4, 119.5, 115.5, 70.8, 70.4, 64.9, 64.8, 56.0, 55.9, 55.0, 54.5, 45.6 × 2, 35.7, 28.4, 18.8, 11.3. HRMS (*m*/*z*) (NALDI-TOF) calcd. for $C_{26}H_{40}O_8N_2S_3Na$ [M + Na]$^+$: 627.1844; found: 627.1840.

Synthesis of vinyl sulfone biotin 5. A solution of biotin (120 mg, 0.49 mmol) in Cl_2SO (5 mL) was kept at rt for 1 h. The reaction mixture was then evaporated under vacuum and co-evaporated with toluene (3 × 15 mL) to give biotin acid chloride. The crude acid chloride was dissolved in anhydrous THF (15 mL) and added dropwise to a solution of **2** (150 mg, 0.40 mmol) and Et_3N (114 μL, 0.80 mmol) in anhydrous THF (10 mL). The new reaction mixture was kept at rt for 10 min. Evaporation of the solvent yielded a crude that was purified by column chromatography (AcOEt–MeOH 10 : 1 → 5 : 1) giving **5** as a syrup (152 mg, 63%). ν_{max}(film)/cm^{-1}: 3354, 3258, 2923, 2870, 1696, 1622, 1457, 1287, and 1121; ^{1}H-NMR (CD_3OD, 400 MHz): δ 6.93 (m, 1 H), 6.34 (m, 1 H), 6.18 (m, 1 H), 4.50 (m, 1 H), 4.33 (m, 1 H), 3.91 (m, 4 H), 3.66 (m, 4 H), 3.52 (m, 2 H), 3.42-3.24 (m, 8 H), 2.94 (m, 1 H), 2.72 (d, 1H, *J* 12.7 Hz), 2.46 (m, 2H), 1.76-1.46 (several m, 8H), 1.26 (m, 3 H), 0.90 (m, 3 H); ^{13}C-NMR (CD_3OD, 75 MHz): δ 178.5, 168.8, 142.3, 132.5, 74.2, 74.1, 68.5, 68.4, 66.1, 64.4, 59.7, 59.1, 58.5, 57.4, 57.1, 43.8, 38.2, 37.0, 32.6, 32.5, 31.5, 29.1, 22.4, 22.3, 14.3. HRMS (MALDI-TOF) calcd. for $C_{24}H_{43}N_3O_8S_3Na$ [M + Na]$^+$: 620.2109; found: 620.2110.

Synthesis of 2-chloroethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside (7). To a solution of 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose **6**[23] (11.7 g, 30 mmol) and thiourea (2.53 g, 33 mmol) in dry acetonitrile (70 mL) was added $BF_3{\cdot}Et_2O$ (8 mL, 63 mmol). The reaction mixture was refluxed for 3 h. After cooling 1-bromo-2-chloroethane (5.9 mL, 69 mmol) and Et_3N (13.5 mL, 90 mmol) were added and the reaction mixture was magnetically stirred at rt for 12 h. Evaporation under vacuum of the solvent gave a crude that was dissolved in CH_2Cl_2 (200 mL) and washed with water (100 mL). The organic phase was dried (Na_2SO_4). Evaporation of the solvent yielded a crude that was purified by column chromatography (CH_2Cl_2 → EtOAc–hexane 1 : 1) giving **7** as a solid (10.6 g, 83%). M.p. 100–102 °C (lit[22] 97–98 °C); $[\alpha]_D$ −30° (*c* 1, chloroform) [lit[22] −38° (*c* 0.7, chloroform)]; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 5.24 (t, 1H, *J* = 9.3 Hz), 5.07 (t, 1H, *J* = 9.7 Hz), 5.03 (t, 1H, *J* = 9.8 Hz), 4.55 (d, 1H, *J* = 10.0 Hz), 4.30-4.10 (m, 2H), 3.80-3.60 (m, 3H), 3.09 (ddd, 1H, *J* = 14.0, 9.8 and 6.1 Hz), 2.90 (ddd, 1H, *J* = 14.1, 9.4 and 6.6 Hz), 2.10, 2.06, 2.04, 2.01 (4 s, 12H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 169.4, 83.9, 76.1, 76.7, 69.8, 68.3, 62.1, 43.4, 32.7, 20.7, 20.6; HRMS (*m*/*z*) (FAB+) calcd. for $C_{16}H_{23}ClO_9S$ + Na: 449.065; found 449.063.

Synthesis of 2-chloroethyl-1-thio-β-D-glucopyranoside (8). To a solution of **7** (3 g, 7.03 mmol) in methanol (60 mL) was added Et_3N (15 mL). The reaction was kept at 40 °C for 8 h. Evaporation under vacuum yielded a crude product that was purified by column chromatography (AcOEt–MeOH 9 : 1) yielding **8** (1.56 g, 86%) as a solid. M.p. 88–90 °C; $[\alpha]_D$ −42° (*c* 1, methanol); ^{1}H NMR (CD_3OD, 300 MHz): δ 4.42 (d, 1H, *J* = 9.7 Hz), 3.86 (dd, 1H, J = 12.0 and 1.2 Hz), 3.80-3.60 (m, 5H), 3.18 (t, 1H, *J* = 8.7 Hz), 3.10 (ddd, 1H, *J* = 14.0, 9.6 and 6.0 Hz), 2.96 (ddd, 1H, *J* = 13.9, 9.6 and 6.2 Hz); ^{13}C NMR (CD_3OD, 75 MHz): δ 87.4, 82.1, 79.6, 74.4, 71.5, 62.9, 44.8, 33.7; HRMS (*m*/*z*) (FAB+) calcd. for $C_8H_{15}ClO_5S$ + Na: 281.0226; found: 281.0222.

Synthesis of 2-ethenyl 1-thio-β-D-glucopyranoside-*S*,*S*-dioxide (9). Compound **8** (1.56 g, 6.02 mmol) was dissolved in a mixture of AcOH–H_2O_2 (2 : 1, 45 mL) and kept in the dark for 2 d. Lyophilization gave a crude that was dissolved in acetone (60 mL). K_2CO_3 (2.4 g, 17.5 mmol) was then added and the resulting suspension was refluxed for 8 h. Evaporation of the solvent was followed by purification by column chromatography (AcOEt–MeOH 10 : 1) yielding a compound that was lyophilized giving **9** (0.5 g, 37%) as a syrup. $[\alpha]_D$ −8.9° (*c* 1, water), $[\alpha]_{436}$ = −16° (c 1, water); ^{1}H NMR (MeOH-d_4, 300 MHz): δ 6.97 (dd, 1H, J = 16.7 and 10.0 Hz), 6.43 (d, 1H, *J* = 16.7 Hz), 6.30 (d, 1H, *J* = 10.0 Hz), 4.78 (s, 3H), 4.34 (d, 1H, *J* = 9.5 Hz), 3.86 (dd, 1H, J = 12.5 and 2 Hz), 3.69 (t, 1H, *J* = 9.1 Hz), 3.68 (dd, 1H, J = 12.6 and 5.3 Hz), 3.45 (t, 1H, *J* = 8.8 Hz), 3.40 (ddd, 1H, *J* = 9.6, 5.5 and 2.1 Hz), 3.34 (s, 1H), 3.31 (t, 1H, *J* = 9.3 Hz); ^{13}C NMR (MeOH-d_4, 75 MHz): δ 136.2, 132.4, 92.6, 82.8, 78.8, 71.0, 70.5, 62.4; HRMS (*m*/*z*) (FAB+) calcd. for $C_8H_{14}O_7S$ + Na: 277.0358; found: 277.0356.

Synthesis of vinyl sulfone ferrocene (11). To a deoxygenated solution of ferrocene methanol **10** (400 mg, 1.85 mmol) in anhydrous THF (30 mL) were added NaH (89 mg, 3.71 mmol) and

DVS (0.46 mL, 4.635 mmol). The reaction mixture was kept at rt for 1 h. AcOH was added to destroy the NaH excess. Evaporation of the solvent under vacuum yielded a crude that was purified by column chromatography (hexane–AcOEt 7 : 1) giving **11** (509 mg, 83%) as an orange solid. M.p. 67.0 °C; IR (ν_{max}) (film): 3099, 2921, 2857, 1638, 1313, 1128, 1099, 816, 754 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 6.67 (dd, 1H, J = 16.6 and 9.9 Hz); 6.36 (d, 1H, J = 16.7 Hz); 6.03 (d, 1H, J = 9.9 Hz); 4.29-4.16 (m, 11H); 3.82 (t, 2H, J = 5.0 Hz); 3.18 (t, 2H, J = 5.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 137.8, 128.7, 82.3, 69.7, 69.5, 68.8, 68.6, 63.2, 55.1; HRMS (FAB+) calcd. for $C_{15}H_{18}FeO_3S$ + H: 335.0404; found: 335.0403.

Synthesis of 3-(methylamino)-propyl silica (18). A suspension of commercial silica gel (Merck 70-230 mesh, ASTM) (5 g), previously activated by thermal treatment (120 °C under vacuum for 24 h), in toluene (25 mL) containing 3-[(methylamino)-propyl]-trimethoxysilane (1.250 g) was heated under reflux for 2 h. After partial evaporation of the solvent under vacuum to approximately 80% of the original volume, the reaction mixture was refluxed for an additional hour. The resulting functionalized silica **18** was filtered, washed with toluene and dried under vacuum at 50 °C.

Synthesis of vinyl sulfone-functionalized silica (19). To a suspension of the amine functionalized silica **18** (1.53 g) in THF–isopropanol (1 : 2, 10 mL) was added DVS (0.640 mL). The mixture was kept at rt for 16 h. The resulting vinyl sulfone–silica **19** was filtered, washed successively with MeOH and CH_2Cl_2 and dried under vacuum at 50 °C.

Protein labeling and bioconjugation assays

Labeling of ConA and avidin with vinyl sulfone-derivatized rhodamine B (3) and dansyl (4) (conjugates 12 and 13). ConA (195 nmol) and avidin (140 nmol) were incubated with the vinyl sulfone-derivatized fluorescent labeling agents **3** (578 nmol, 1 : 4 stoichiometry, and 1154 nmol, 1 : 8 stoichiometry) and **4** (990 nmol, 1 : 5 stoichiometry, and 1980 nmol, 1 : 5 and 1 : 10 stoichiometry), respectively, in 50 mM HEPES pH 8 at 37 °C for 24 h. Samples were dialyzed first against 100 mM HEPES pH 8 supplemented with ethanolamine (2 × 100 mL) to block the excess of vinyl sulfone-derivatized reagents and then against 100 mM HEPES pH 8 (7 × 100 mL) for 4 d. The extent of labeling was monitored at 3, 8 and 24 h by taking aliquots of 50 µL and blocking by addition of 50 µL of SDS-PAGE loading buffer prior denaturing at 100 °C for 4 min. The samples were not dialyzed.

Glycosylation of commercial HEW lysozyme with glucopyranosyl vinyl sulfone 9 (conjugate 15). The reaction was carried out by mixing and stirring 500 µL of 50 mg mL^{-1} HEW lysozyme in 90 mM phosphate buffer pH 7.7 and 10% (v/v) 2-propanol with 10.5 mg glucopyranosyl vinyl sulfone **9** for 72 h at rt. To remove the excess of **9** and stop the reaction, the mixture was transferred to a 3500 kDa cutoff membrane (Spectrum) and dialyzed against a total volume of 1500 mL of 20 mM acetate buffer at pH 4.5 in three steps. The reaction was monitored every 24 h by native PAGE run with a BioRad apparatus with reversed polarity and stained with Coomassie Brilliant Blue. For the crystallization procedure and the determination of the tridimensional structure see the ESI.†

Labeling of HEW lysozyme with vinyl sulfone rhodamine B (3) at different pH and temperatures (conjugate 14). The labeling of 11.2 nmols of HEW lysozyme with 288 nmols of vinyl sulfone rhodamine B (**3**) was carried out by incubation at rt or 30 °C for 64 h in 160 mM buffer pH 5 and 6 (acetate buffer), or 7 and 8 (HEPES buffer). The samples were then dialyzed against distilled water for 24 h and analyzed by MALDI-TOF (Fig. 3).

Labelling of BSA with ferrocene vinylsulfone 11 (conjugate 16). To three aliquots (2 mL) of a 2 mg mL^{-1} solution of BSA in 50 mM HEPES buffer at pH 8.5 were added the ferrocene vinyl sulfone derivative **11** (0.2, 1.0 and 2.0 mg) dissolved in DMSO (0.1 mL) to get a 1 : 10, 1 : 50 and 1 : 100 BSA : **11** molar ratio, respectively. The samples were kept at rt for 24 h. Centrifugation was followed by dialysis against an 85 mM phosphate buffer at pH 7.0. BSA concentration was determined by Bradford's method[24] and Fe content was spectrophotometrically determined.[16]

Biotinylation of BSA (conjugate 17) and interaction with dansyl–avidin (conjugate 13). BSA (75 nmols) and vinyl sulfone-derivatized biotin **5** (370 nmols) were incubated in 600 µL of 50 mM HEPES pH 8.0 at 37 °C for 24 h. The resulting conjugate **17** was dialyzed first against 100 mM HEPES pH 8, supplemented with ethanolamine (2 × 100 mL) to block the excess of **5**, and then against 100 mM HEPES pH 8 (7 × 100 mL) for 4 d. The concentration of protein was determined by Bradford's method.[24] The formation of BSA–biotin–avidin–dansyl complexes was achieved by the incubation in 33 mM HEPES pH 8.5 at rt for 30 min of 0.15 nmols of tetrameric avidin with either 0.15 or 6 nmol of biotin–BSA to yield 1 : 1 and 1 : 4 dansyl–tetrameric avidin : biotin–BSA complexes respectively. SDS-PAGE loading buffer was added and the samples were denatured in mild conditions by heating at 100 °C for 2 min.

Labeling of BSA and lysozyme with vinyl sulfone-derivatized rhodamine B 3 as a pre-stain dye. BSA and lysozyme (33 µg), and **3** (3.6 mmol) were incubated in 222 µl of 75 mM HEPES pH 8.8 at 100 °C for 5 min prior mixing with the SDS-PAGE loading buffer and denaturing at 100 °C for 4 min.

Electrophoresis of the labeled proteins. Samples analyzed by SDS-PAGE[25] in a 12% polyacrylamide gel (12% T : 1.3% C) in a MiniProtean 3 (BioRad). The fluorescence was detected by exciting at 365 nm with a conventional UV transilluminator (Genesys Inst.) and the gels were stained by either Coomassie Brilliant Blue[26] or silver stain.[27]

Immobilization assays

Immobilization assays of invertase with bioconjugable vinyl sulfone silica (19) and enzymatic activity determination. Vinyl sulfone silica **19** (0.5 g) was suspended in 5 mL of a 47 mg mL^{-1} solution of invertase from *Saccharomyces cerevisiae* in 244 mM phosphate buffer pH 7.5 and then 2.88 mL of water. The samples were prepared in duplicate and incubated at either 4 °C or rt. The reaction was allowed to proceed for 30 h (until there was no variation in A_{280} between two consecutive measurements of the protein in solution), and then the samples were washed with 100 mM phosphate buffer at pH 7.5 (6 × 5 mL) and 2 M NaCl in 100 mM phosphate buffer pH 6.5 (2 × 5 mL + 2 × 10 mL). The enzymatic activity of the immobilized invertase was monitored by analysis of the optical rotation with a Perkin-Elmer polarimeter.

The immobilized enzyme was incubated with a 3% (w/v) solution of sucrose in 100 mM phosphate buffer pH 7.5 and the optical rotation was measured 5 times within 17 h. The values were fitted by linear regression (coefficient of determination >0.9), the slope multiplied by 1000 being the enzymatic activity in arbitrary units. Results are summarized in Table 3.

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