Vinyl Sulfone: A Multi-Purpose Function in Proteomics

F. Javier Lopez-Jaramillo, Fernando Hernandez-Mateo
and Francisco Santoyo-Gonzalez
Instituto de Biotecnologia,
Universidad de Granada, Granada
Spain

1. Introduction

The outstanding development attained in the actual state-of-the-art on Proteomics has been reached not only by the integration of a panel of sophisticated analytical and bioinformatics techniques and instrumentations but also by the intelligent application of classical and advanced synthetic methodologies used in protein chemistry (Lundblad, 2005; Tilley *et al.*, 2007). Covalent modification of proteins is a powerful way to modulate their macromolecular function. Nature accomplishes such alterations through a range of post-translational modifications that in turn mediate protein activity. Artificial covalent modification of proteins is an arduous but fruitful task of major interest for the biophysics and biochemistry communities that normally pursue as goals the detection or purification of the protein itself in order to have a more thorough understanding of molecular mechanisms and the expansion of the applicability of such biomolecules. Despite the intrinsic difficulties associated to perform those chemical modifications of proteins, the attachment of analytical or engineered probes for protein tracking (labelling) (Giepmans *et al.*, 2006; Waggoner, 2006; Wu & Goody, 2010) or protein profiling (chemical proteomics) (Evans & Cravatt, 2006; Cravatt *et al.*, 2008), the introduction of affinity tags for separation-isolation of proteins (affinity chromatography) (Azarkan *et al.*, 2007; Fang & Zhang, 2008) or for mass spectroscopy-based protein identification and characterization (chemical tagging) (Leitner & Lindner, 2006), the immobilization onto solid supports (microarray technologies) (Wong *et al.*, 2009) and the conjugation with other biomolecules (post-translational modifications) (Gamblin *et al.*, 2008b; Walsh, 2009; Heal & Tate, 2010) are among some of the most useful and frontier techniques and methodologies used in Proteomics.

For the chemical modification of proteins, a large number of strategies are nowadays available (Hermanson, 2008). The straightforward and probably most used of those strategies takes advantage of the chemical reactivity of the endogenous amino acid side chains commonly by using the nucleophilic character of some of them in a nucleophile-to-electrophile reaction pattern that leads to specific functional outcomes (Baslé *et al.*, 2010). This classical residue-specific modification chemistry, however, is rarely sufficiently selective to distinguish one residue within a sea of chemical functionality and for this reason more intricate approaches have been developed in recent times to introduce a unique chemical handle in the target protein that is orthogonal to the remainder of the proteome

(Hackenberger & Schwarzer, 2008). Direct incorporation of non-canonical amino acids into proteins via the subversion of the biosynthetic machinery is an attractive means of introducing selectively new functionality by either a site-specific or residue-specific manner (Beatty & Tirrell, 2009; de Graaf *et al.*, 2009; Johnson *et al.*, 2010; Liu & Schultz, 2010; Voloshchuk & Montclare, 2010; Young & Schultz, 2010) that in combination with recent and notorious advances in bioorthogonal reactions (nucleophilic addition to carbonyl, 1,3-dipolar cycloaddition reactions, Diels-Alder reactions, olefin cross-metathesis reactions and palladium-catalyzed cross-coupling reactions) has allowed an exquisite level of selectivity in the covalent modification of proteins (Wiltschi & Budisa, 2008; Sletten & Bertozzi, 2009; Lim & Lin, 2010; Tiefenbrunn & Dawson, 2010). In spite that major technical challenges have been overcome, a prodigious amount of lab work and the concurrently optimization of a larger set of parameters is normally required for those advanced and selective methodologies in comparison with conventional organic reaction development.

In this general frame, the purpose of the present chapter is to provide a general outlook on the applications on Proteomics of a particular methodology, the vinyl sulfone chemistry (Simpkins, 1990; Forristal, 2005; Meadows & Gervay-Hague, 2006), with a particular emphasis in some recent advances that illustrate the multi-purpose character of this chemical function in this field. Vinyl sulfones readily forms covalent adducts with many nucleophiles ("hard" and "soft") via a Michael-type 1,4-addition. Two prominent characteristics of this reactive behaviour have allowed its implementation on Proteomics: the possibility to perform those reactions in physiological conditions (aqueous media, slightly alkaline pH and room temperature) that preserves the biological function of the proteins and the absence of catalysts and by-products. In addition, the introduction of the vinyl sulfone is not a difficult task and the resulting functionalized reagents or intermediates are stable.

The chapter is organized in three sections. In a first instance, a general overview of the vinyl sulfone chemistry in terms of the most relevant methods of synthesis and aspects of their reactivity will be followed by a discussion of the application of this chemical behaviour with proteins. Their advantages and disadvantages with other currently available methodologies to modify amine and thiol groups naturally present in proteins will be compared. In a second section the applications of vinyl sulfones to Proteomics will be enumerated. Finally, the wide scope of the vinyl sulfone chemistry in other omic sciences will be discussed.

2. Vinyl sulfone chemistry

Vinyl sulfones (α,β-unsaturated sulfones) are productive and widely used intermediates in organic synthesis that also have a remarkable biomedical significance owed to their capability to act as irreversible inhibitors of many types of cysteine proteases through conjugate addition of the thiol group of the active site cysteine residue. This feature is the basis of some modern applications of this chemical function to Proteomics as it will be discussed below (section 3.2). Currently, there exists a solid body of knowledge on the chemical reactivity of the vinyl sulfone that allows the functionalization of any organic substrate.

2.1 Synthesis of vinyl sulfones

Vinyl sulfone is a functional group accessible by a broad variety of traditional synthetic methods and other contemporary reactions that have been comprehensively reviewed (Simpkins, 1990; Forristal, 2005; Meadows & Gervay-Hague, 2006).

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For Proteomics, the most relevant of these procedures are those that used 2-halo or 2-hydroxyethylthioethers as starting materials (Fig. 1). From these compounds formation of a vinyl sulfone is feasible by three alternative strategies: sequential elimination and oxidation in either order (routes a and b) or simultaneous oxidation-elimination (route c). When the elimination step is firstly performed (route a), the vinyl thioether intermediate obtained can be easily oxidized to the corresponding vinyl sulfones by common oxidizing agents (H_2O_2-acetic acid, m-chloroperbenzoic acid –mCPBA- or periodic acid -HIO_4-) or the commercial Oxone® reagent. The slow kinetic showed by the method based in H_2O_2 (Bordwell & Pitt, 1955) has been overcome by the concomitant use of some catalysts ($MnSO_4$ or tetrakis(pentafluorophenyl)porphyrin) in order to exploit the goodness of this methodology: low cost and toxicity, and high yields (Alonso *et al.*, 2002; Baciocchi *et al.*, 2004).

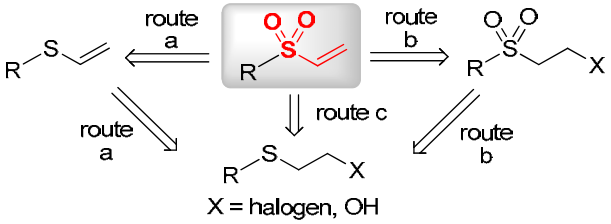

Fig. 1. General retrosynthetic pathway for the synthesis of vinyl sulfones from 2-halo or 2-hydroxyethylthioethers

In the alternative sequence (route b), the sulfone is obtained previously by using the reagents just mentioned followed by the elimination step that is favoured by the strong electron-withdrawing effect of the sulphur function, being only necessary a weak base (triethylamine) in case of the dehydrohalogenation (Brace, 1993) or the conversion on a good leaving group, usually a sulfonic ester, in the dehydration option (Lee *et al.*, 2000; Galli *et al.*, 2005).

On the other hand, ammonium molybdate in the presence of H_2O_2 or ozone allows the formation of vinyl sulfones in one-step from derivatized ethylthioethers with satisfactory results (route c) (Krishna *et al.*, 2003).

In addition to the methodologies commented, the ionic and radical addition to unsaturated compounds (alkenes, alkynes and allenes), the addition of sulfonyl-stabilized carbanions to carbonyl compounds, the manipulation of acetylenic sulfones and the use of organometallic reagents are other routes for the synthesis of vinyl sulfones (Simpkins, 1990; Forristal, 2005; Meadows & Gervay-Hague, 2006) that in practice have found limited applications in Proteomics up to the present.

2.2 Reactivity of vinyl sulfones

Vinyl sulfones as sulfonyl-containing compounds readily undergo a variety of cycloaddition reactions and conjugate additions as excellent Michael acceptors because of the electron poor nature of their double bond owed to the sulfone's electron withdrawing capability that make them good electrophiles. The cycloadditions reactions have been reviewed in detail (De Lucchi & Pasquato, 1988; Simpkins, 1990; Forristal, 2005) but their applications in Proteomics have been null. For this reason these relevant reactions are considered out of the scope of the present chapter and an interested reader is referred to those articles. However,

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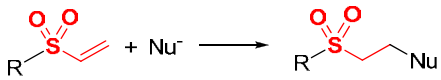
conjugate additions to vinylsulfones involving both "hard" and "soft" nucleophiles are of paramount importance in Proteomics, and for this reason a general outlook of this sort of reactions is given.

A significant body of work has been devoted to the conjugate additions of vinyl sulfones with carbon nucleophiles with both non-stabilised organometallics and stabilised anions (including enolates). In addition, vinyl sulfones have been widely exploited as acceptors in radical conjugated additions with a variety of nucleophilic radicals (Srikanth & Castle, 2005) and have been used in organocatalytic methodologies where they have demonstrated their versatility and power in asymmetric reactions for the construction of carbon-carbon bonds with exceptional levels of enantioselectivity (Alba *et al.*, 2010). Aside from these reactions with carbon nucleophiles, heteroatomic nucleophiles involving nitrogen, sulphur and oxygen can participate efficiently in conjugate addition reactions with vinyl sulfones in a protic environment where the incipient carbanion is quickly quenched. In these reactions, base catalysts are often unnecessary for amines because of the strong nucleophility of the nitrogen atom. However, although thiols are generally more nucleophilic than amines, weak bases are often used to deprotonate them due to their comparatively higher acidity (Bednar, 1990).

All the conjugate additions with vinyl sulfones share a similar reaction pattern by addition at the β-position of the sulfone and, on this basis, these reactions are a well-established method of creating β-heterosubstituted sulfones (Fig. 2). In all cases, the resulting 1,4-addition products contain either the sulfonyl moiety which can undergo subsequent functional group transformations or can be easily removed (by means of Mg or Hg/Na) making these compounds a perfect choice to afford easily naked alkyls (Nájera & Yus, 1999).



Nu^- = C-, N-, and S-Nucleophiles

Fig. 2. General conjugated Michael-type addition of vinyl sulfones and nucleophiles

Heteroatomic nucleophiles differ in the kinetic of their conjugate addition to Michael acceptors including vinyl sulfones, fact that is relied to their nucleophilicity. Studies on model compounds, including amino-acids, were performed to evaluate the influence on the reaction rates of these α,β–unsaturated compounds of different factors either inherent to the nucleophiles (charge, electronic structure and size) or depending on the environment (interactions with neighbouring ionisable groups, steric factors and pH) (Friedman *et al.*, 1965; Morpurgo *et al.*, 1996; Lutolf *et al.*, 2001). As a general rule, it is observed a direct correlation between the reaction rates and the anion concentration which is determined by the pK_a values and the pH of the medium in such a way that rates increase with pH due to the increased concentration of the anion. However, comparative studies performed in these pioneering contributions concerning the relative nucleophilic reactivities of amino groups and mercaptide ions showed that at comparable pK_a values and steric environments vinyl sulfones react with thiols significantly quicker than with amines or other nucleophiles. From these results, it has been assumed that vinyl sulfones are selective in the reactions with thiol groups relative to reaction with amino groups providing that the reaction is not carried out at alkaline pH. The implementation of these observations in protein chemistry is on the rationale behind numerous chemoselective modifications of cysteine-containing peptides

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and proteins by the Michael-type addition reaction of vinyl sulfone derivatives. However, given the multifunctional character and complexity of proteins, the preference of vinyl sulfones for thiol groups should be considered with precaution as recent findings have demonstrated (*vide infra* section 2.3.).

Considering that selectivity is a key point in bioconjugation and particularly in Proteomics, the next section is devoted to give a general overview of the different strategies currently available for the modification of the side groups of amino and thiol-containing amino acids to put in context vinyl sulfone-based strategies in relation with those methodologies.

2.3 Vinyl sulfones and other methodologies for chemical modification of proteins at amino and thiol-containing residues

The most popular but one of the least site-specific and residue-specific strategies for modification of proteins targets the lysine residue because of its predominant presence (up to 6% of the overall amino acid sequence, the 11th most frequent residue) (Villar & Kauvar, 1994; Villar & Koehler, 2000; UniProtKB/TrEMBL database, 2011-06), 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.

Although the primary amine group of lysine is protonated under physiological pH, it can still react as a nucleophile (Fig. 3). Amine reactive electrophilic reagents used with proteins are usually acylating agents, such as succinimidyl esters, sulfonyl chlorides and isothiocyanates (**1**, **2** and **3**, respectively). However they are not exempt of drawbacks. Succinimidyl esters (**1**) are the best suited amine reactive compounds as they react with lysines without exogenous reagents such as bases. More soluble but less reactive sulfosuccinimidyl esters have been used to overcome their poor water solubility (Staros *et al.*, 1986). Sulfonyl chlorides (**2**) are highly reactive but are also unstable in water (Lefevre *et al.*, 1996), specially 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 imidazole (histidine). Isothiocyanates (**3**) are stable in water although their reactivity is only moderate and the degradation of the resulting thiourea has been reported (Banks & Paquette, 1995). In addition, the optimal pH needed for the reaction with lysine of these reagents (pH 9-9.5) is higher than for the formation of succinimidyl esters (pH 8-9) and may be unsuitable for modifying alkaline-sensitive proteins.

Other approaches are: a) the reductive amination of an aldehyde (**4**) using water compatible hydrides, a two-step procedure that make this route more challenging (Jentoft & Dearborn, 1979); b) the amidination with imidoesters (**5**) at elevated pH (~9) or with iminothiolane (**6**, Traut's reagent) near pH 8, reagents that conserve the overall charge of the side group (Means & Feeney, 1990), and c) the use of thioesters or dithioesters (**7**, X=O or S, respectively), being these last mild reagents for lysine residues in the absence of competing cysteine residues (Wieland *et al.*, 1953) that reacts very fast, specifically and irreversibly although they have a limited solubility in water.

In contrast with lysine, cysteine residues are perhaps the most convenient target of the proteogenic amino acids for selective modification of proteins owing to their low natural abundance (the second less abundant amino acid in proteins with a frequency of 1.36%) (Villar & Kauvar, 1994; Villar & Koehler, 2000; UniProtKB/TrEMBL database, 2011-06) and the strong nucleophilic character of the sulfhydryl side chain higher than a primary amine, especially at pH below 9, that results in a general kinetic selective modification of cysteine over lysine residues. Despite thiols often form disulphide

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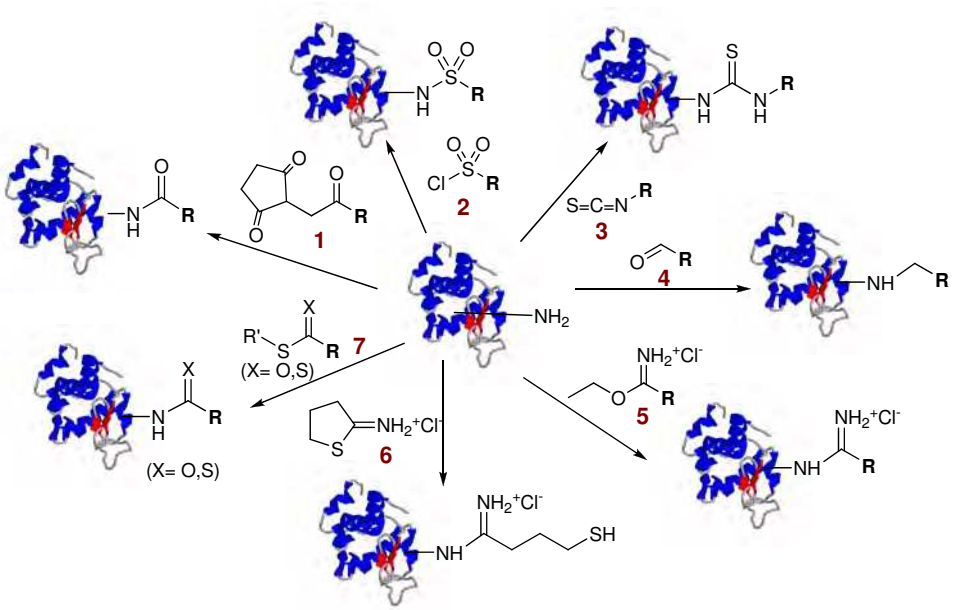

Fig. 3. Reagents for the chemical modification of Lysine residues in proteins

oxidized dimers, the enduring utility of this amino acid in protein modification is evidenced for the wide panel of methodologies that allow the mild, selective, rapid and quantitative reaction at cysteine and their derivatives under appropriate conditions in either a reversible or irreversible way (Fig. 4) (Chalker *et al.*, 2009). Direct alkylation methods with a variety of electrophilic reagents such as α–halocarbonyls (**8**, iodoacetamide), Michael acceptors (including maleimides **9**, vinyl sulfones **10** and related α,β-unsaturated systems) and β–haloethylamine (**11**) (Lindley, 1956) are common techniques for cysteine modification. More specific reactions of the sulfhydryl groups that do not interfere with other amino acids are oxidation and desulfuration of cysteine. Protein modifications via oxidative disulfide bond formation is one of the simplest methods that can be accomplished by simple air oxidation, disulfide exchange with Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) -DTNB-) or some others activated reagents (iodine or sulfenyl halides) (**12-14**) (Anson, 1940; Fontana *et al.*, 1968). Desulfurization at cysteine may involve its transformation into a thioether, the reductive removal of the thiol group to yield alanine (Yan & Dawson, 2001) or the oxidative elimination of cysteine to yield dehydroalanine (Bernardes *et al.*, 2008), which behaves as a Michael acceptor with thiol nucleophilic reagents. Finally, some metal-mediated reactions (cross-metathesis and Kirmse-Doyle reactions) performed in ally sulfide derivatives have recently extended the panoply of chemical modifications at cysteine (Lin *et al.*, 2008).

However, cysteine modification is not exempt of some drawbacks because besides the low frequency of cysteine in proteins and its relevance for the function, the difference of nucleophility between amine and thiol groups in proteins is dependent on surrounding residues (Bednar, 1990), the selectivity being compromised, and the use of specific reaction on the sulfhydryl group may be limited by the compatibility of the reaction conditions with the functionality of the protein.

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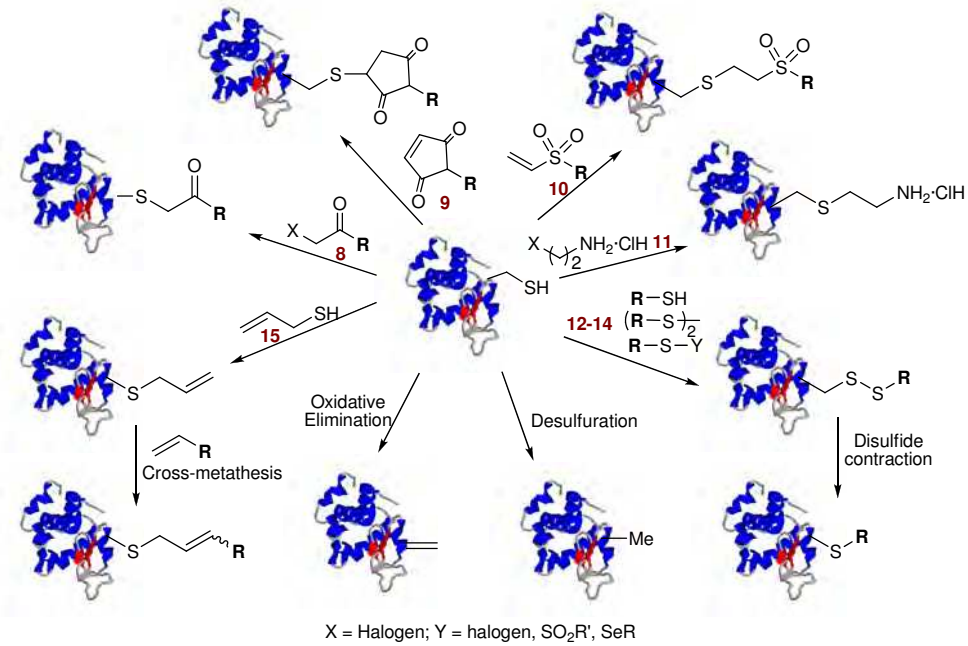

Fig. 4. Reagents for the chemical modification of Cysteine residues in proteins

In this context, the excellent capability of vinyl sulfones to act as Michael acceptors has been used but not fully exploited up to the present for protein modification, despiste attractive characteristics offered by this methodology such as water stability of the sulfur function for extended periods, particulary at neutral pH where they are resistant to hydrolysis, the lack of by-products in conjugated reactions, the needless use of organometallic catalysts, and the stability of the linkages formed.

It is generally accepted that i) the larger nucleophilic character of thiol makes cysteine residues the preferential target of vinyl sulfone derivatized reagents, ii) the ε-amino groups of lysine and to a lesser extent the imidazole ring of histidine side chain are secondary targets and iii) the pH of the reaction medium may be use to control the relative reactivity of these funtional group (Friedman & Finley, 1975; Masri & Friedman, 1988). Studies on the reactivity of poly(ethylene glycol) vinyl sulfone toward reduced ribonuclease (Morpurgo *et al.*, 1996) found that the reaction with cysteine groups is rapid and selective at pH 7-9 and with lysines proceeds slowly at pH 9.3. Other residues were described as not reactive. These results have been the dogma of the reactivity of vinyl sulfone with proteins. However, as early as 1965, it was reported that at comparable pKa values and steric enviroment thiols are 280 times more reactive than amine groups but also that the reactivity of the thiol group in an aminothiol acid is influenced by the presence of charge on neighboring amino groups and caution in the use of specific sulfhydryl specific reagents in proteins was recommended (Friedman *et al.*, 1965). A systematic study of the thio Michael additions confirmed the importance of the charges close to the cysteine and the existence of a linear correlation between thiolate concentration and kinetic constants (Lutolf *et al.*, 2001). More recently, the authors' group also found unexpected reactivity of His at pH 7.7 in the reaction of lysozyme

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with sugar vinyl sulfone derivatives and a double addition to a single Lys while other Lys residues remained unreacted (Lopez-Jaramillo *et al.*, 2005). In fact, the reaction of lysozyme proceeds very fast even at pH 5. At this point, it is important to recall that the non equivalence of identical residues present in proteins is an important concept frequently overlooked. The different nucleophilic character of identical residues is a well illustrated concept. Thus, it has been reported pKa values for internal lysines as low as 5.3 (Isom *et al.*, 2011), the standard pKa being ~10.4, and also pKa values for histidine ranging from 9.2 (His72 in tyrosine phosphatase) to 4.6 (His40 of bovine chymiotrypsinogen), the standard pKa value being 6.6 (Edgcomb & Murphy, 2002). Thus, the presence of a plethora of potential reactive groups in proteins and the dependence of their reactivity on the neighboring residues make group-specific modification chemistry unsuited as a general strategy for the selective modification of a particular residue but still valid for many omics applications.

Finally, it should be mentioned that in comparison with maleimides, one of the most widely-used conjugated reagent for chemical modifications of thiol-containing proteins, vinyl sulfones offers as advantages the aforementioned enhanced stability in aqueous alkaline conditions and the fact that the reaction product is a single stereoisomer, unlike conjugation with maleimides, which produces two potential stereoisomers.

3. Application of vinyl sulfones in proteomics

Vinyl sulfones have found application in most of the subdomains of modern Proteomics. Overall, these applications can be group in two main areas: labeling in their different variants (attachment of analytical or engineered probes for protein tracking, protein identification or protein profiling) and immobilization with different purposes (affinity chromatography and microarray technologies), two of the cornerstones of any omic science. In addition, vinyl sulfones have found applications in the conjugation of proteins with other biomolecules to yield post-translational modifications.

3.1 Vinyl sulfone-based labeling and chemical tagging

Proteomic often requires labeling of compounds for detection/isolation. Mass spectrometry offers a label-less method currently used in quantitative Proteomics. *Stricto sensu* it involves an isotopic labeling, usually referred as isotope tagging (Nakamura & Oda, 2007; Iliuk *et al.*, 2009), that can be carried out *in vivo* in the cell culture by metabolic incorporation or alternatively after protein extraction by chemical labeling, the former highlighting the importance of chemical tagging reactions (Leitner & Lindner, 2006). The reactivity of the vinyl sulfone group toward amino acids naturally occurring in proteins is conceptually an attractive derivatization strategy to promote the covalent attachment of labels to proteins. Despite that bibliographic references are scarce, vinyl sulfone derivatized dyes, fluorophores and other tags (biotin) have been already described and implemented in Proteomics.

The use of vinyl sulfone derivatized reagent for detection in Proteomic dates back to 1972 when Remazol dyes were reported as prestaining reagents during denaturation prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) that also allow the tracking by eye of the migration of the protein during the electrophoretic separation (Griffith, 1972). Remazol dyes are easily converted to vinyl sulfone derivatives at alkaline pH and upon reaction the electrophoretic mobility of the sample is not seriously affected

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since the dodecyl sulfate bound to the protein renders irrelevant any small charge difference in the proteins. Ulterior studies found that pre-stained proteins eluted from the gels retained immunological reactivity and were suitable to raise monospecific antibodies (Saoji *et al.*, 1983). The original idea is still valid and Remazol dyes are currently used in prestained color-coded molecular weight markers for gel electrophoresis (Compton *et al.*, 2002).

In a mass spectrometry-driven proteomic scenario gel electrophoresis is still part of the workflow, the in gel digestion of proteins being a cornerstone (Shevchenko *et al.*, 2006). However, the staining of the gel, selection and extraction, in-gel reduction, alkylation and destain for the subsequent tryptic digestion is a time and labor demanding process that represents a bottleneck. Pre-electrophoresis staining is an attractive approach that has not been extensively used because of the slight mobility differences that have been reported, despite the availability of fluorescent dyes that are charge-matched to preserve the pI of the proteins upon labeling (Miller *et al.*, 2006). In this context, the use of Uniblue A (**16**, Fig. 5), the vinyl sulfone derivative of Remazol Brilliant Blue R colorant, has been proposed as a straightforward strategy that i) yields the covalently stain of both simple and complex protein samples within 1 minute and ii) does not compromised protein profiles on the gels (Mata-Gomez, 2010). Another application in this area proposed the use of the reactivity of divinyl sulfone with the α-amino groups of N-terminal residues in Proteomics since it enhances the abundance of the a1 fragments, defining the N-terminal residue and providing a "one step Edman like information"(Boja *et al.*, 2004).

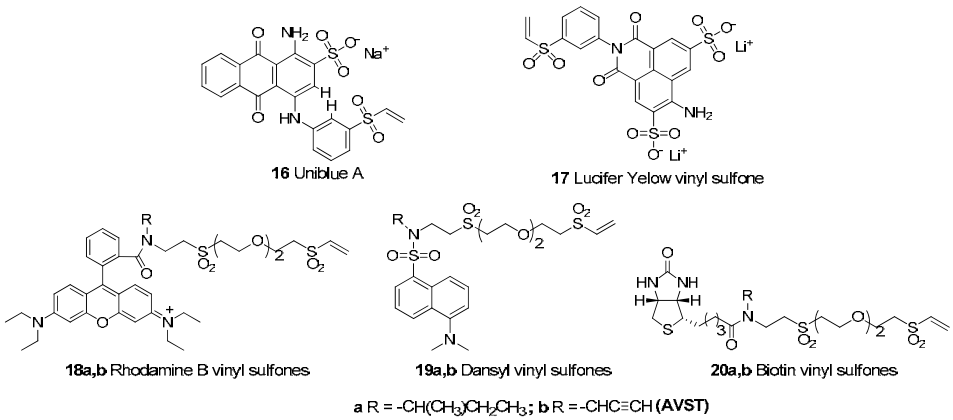

Fig. 5. Some vinyl sulfone derivatized dyes, fluorescent probes and tags (biotin) used in Proteomics

On that concerning fluorophores, to our knowledge Lucifer Yellow vinyl sulfone (**17**, Fig. 5) was the first vinyl sulfone derivatized fluorophore applied to protein studies. This compound was the fluorescent probe used for fluorescence resonance energy transfer experiments on the chloroplast coupling factor 1 that showed that ATP induces changes on the nucleotide binding site and switches properties (Shapiro & McCarty, 1988; 1990) and allowed to gain insight into the asymmetry of the α subunit of CF1 (Lowe & McCarty, 1998). Lucifer Yellow vinyl sulfone was also used to study the interaction between Rod G-protein α subunit and cGMP-phosphodiesterase γ-subunit (Artemyev *et al.*, 1992). More recently the synthesis of vinyl sulfone derivatized rhodamine B and dansyl (**18a** and **19a**,

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Fig. 5) and their reactivity with a pool of commercial proteins has been reported (Morales-Sanfrutos *et al.*, 2010b). The results showed that the protein itself influences the extend of coupling and that the labeling is feasible regardless of isoelectric point, number of potential nucleophiles or presence of glycosylation in the protein. The study also showed the potential of the rhodamine B vinyl sulfone as a prestaining reagent since the labelling does not affect the electrophoretic mobility or post-electrophoresis silver stain. The analysis of the influence of the reaction conditions on the reactivity between vinyl sulfone and Henn Egg-white (HEW) lysozyme revealed 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 coupled to lysozyme. In a later work the same authors developed a series of alkyne vinyl sulfone derivatized tags (AVST reagents) bearing rhodamine B or dansyl (**18b** and **19b**, Fig. 5) and demonstrated their applicability as self-reporter reagents for monitoring the introduction of the alkyne function and their potential to carry out further functionalization in any scenario based on click-chemistry (Morales-Sanfrutos *et al.*, 2010a).

Vinyl sulfone derivatization has been used to attach other tags to proteins such as biotin. The authors' group has described (Morales-Sanfrutos *et al.*, 2010b) the conjugation of biotin vinyl sulfone (**20a**, Fig. 5) to promote the coupling of the biotinylated protein to avidin in the context of what is known as *avidin-biotin technology* (Savage et al., 1992). In the more advanced contribution mentioned above (Morales-Sanfrutos *et al.*, 2010a), the synthesis of vinyl sulfone bifunctional tags bearing simultaneously biotin and a fluorophore as a single-attachment-point reagents (BTSAP, **22** and **23** Fig. 6) was easily performed by click copper-catalyzed azide-alkyne cycloaddition (CuAAC) attachment of the AVST fluorophores reagents (**18-20b**, Fig. 5 and Fig. 6). The combination of vinyl sulfone as reactive group, biotin as an anchor point and a fluorophore as a reporter group in the BTSAP reagents made of versatile compounds with a clear potential in Proteomics as illustrated in the labeling of the low reactive protein horseradish peroxidase (HRP) (Fig. 6, route a). Alternatively, the dual labeling of this protein was also attained by a CuAAC-based sequential approach consisting in the labeling with an AVST reagent and ulterior click conjugation with an azide-containing biotin derivative (**21**) (Fig. 6, route b).

3.2 Vinyl sulfone-based chemical proteomics

Vinyl sulfones have been used in activity-based protein profiling (ABPP) (Evans & Cravatt, 2006; Hagenstein & Sewald, 2006), a methodology of interest in the so-called chemical Proteomics subdomain devoted to measure the activity of proteins to gain insight into the functional role of proteins in cell physiology and pathology. ABPP is a chemical strategy based on the use of activity-based probes (ABPs), small molecules that form activity dependent covalent bonds to a target enzyme (Fig. 7). These probes contain three main elements: (1) a warhead or reactive functional group that forms the covalent bond with the active site catalytic residue of a target (2) a linker that can be used to control the specificity of binding interactions between the probe and target enzyme and (3) a tagging group that allows probe labeled targets to be isolated, biochemically characterized or imaged. The majority of ABPs contain electrophilic warheads derived from well-known irreversible enzyme inhibitors. Many of the most versatile ABPs represent the simple conjugation of well-characterized covalent inhibitors to reporter tags such as fluorophores and biotin. The research efforts performed in this field have engendered ABPP probes for numerous enzyme classes.

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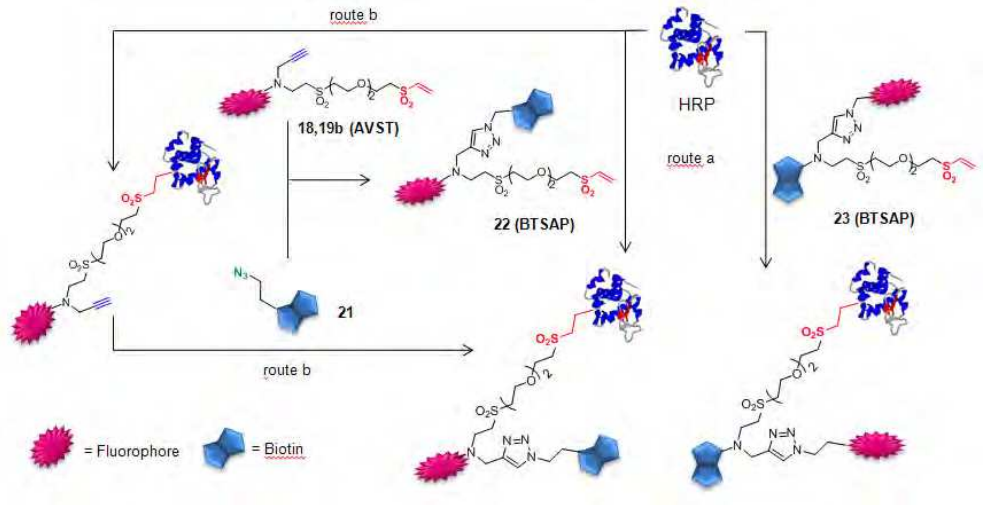

Fig. 6. Vinyl sulfone derivatized bifunctional tag single-attachment-point reagents (BTSAP)

Traditionally vinyl sulfones have been recognized as Cys protease inhibitors (Palmer *et al.*, 1995; Wang & Yao, 2003) and hence a large number of vinyl sulfone-containing peptides have been synthesized and exploited to inhibit them. On this basis, the reactivity of the vinyl sulfone function toward thiols has been used in ABPs to address cysteine proteases. As representative examples of these vinyl sulfone-based ABPs, it can be mentioned the studies performed on deubiquitinating enzymes with ABPs consisting of a truncated ubiquitinn or ubiquitin-like probe and biotin as reporter (**24**, Fig. 7A) (Borodovsky *et al.*, 2005). In addition, aryl vinyl sulfone and sulfonate probes have been developed to investigate the activity of protein tyrosine phosphatases (PTP) (Fig 7B.). In this case an azide group has been incorporated to the tag (**25)** to attach by click chemistry alkyne labels (**26**) such as biotin in order to facilitate the analysis (Liu *et al.*, 2008). Finally, in other studies dipeptidyl peptidase I (i.e. cathepsin C) has been selectively labeled by a vinyl sulfone norvaline-homophenylalanine dipeptide ABP (**27**, Fig. 7C) (Yuan *et al.*, 2006).

Vinyl sulfone-based ABPs have been also used for other class of hydrolases (Fig. 8). Thus, a series of tripeptide and tetrapeptide vinyl sulfone has been used as proteasome-directed ABPs to selectively engage the catalytic threonine nucleophile within proteasome active sites (Bogyo *et al.*, 1998; Nazif & Bogyo, 2001). By varying the peptide portion of the probes in a positional scanning library (**29**), the researchers gained insights into the substrate recognition properties of specific proteasomal subunits, culminating in the development of Z-subunit specific inhibitors that were used to identify this subunit as the principal trypsin-like activity of the proteasome. More recently, azide versions of vinyl sulfone probes (**30**) were used as tag-free ABPs to profile proteasomal activities in living cells, detection accomplished by tandem labeling strategies using highly specific bioorthogonal Staudinger ligation with a phosphine reporter tag (Ovaa *et al.*, 2003).

3.3 Vinyl sulfone-based affinity chromatography applications

Two dimensional gels resolve no more than several thousand proteins, only the most abundant ones being visualized, and Proteomics also includes the analysis of post-

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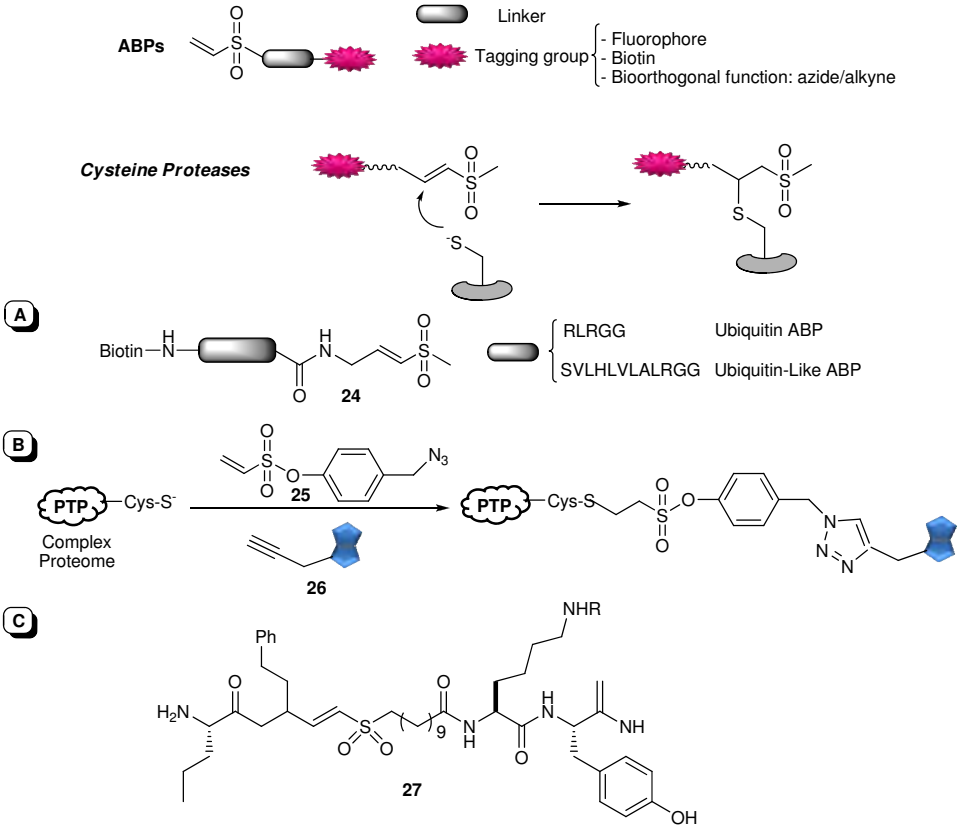

Fig. 7. General structure of vinyl sulfone-based ABPs and representative examples of those targeting cysteine proteases.

translational modifications (Mann & Jensen, 2003) and protein-protein interactions (Blagoev *et al.*, 2003). In this context, the immobilization of ligands plays a central role either for bioseparation and concentration of biomolecules (Lee & Lee, 2004; Azarkan *et al.*, 2007), for pull-down assays and mass spectrometry analysis (Bécamel *et al.*, 2002) or for high-throughput screening in array format (Cahill, 2000). However, examples of vinyl sulfone functionalized supports either for affinity chromatography or arrays are scarce.

Still in use, divinyl sulfone-activated agarose was the first support bearing the vinyl sulfone function to turn it out into an affinity support upon reaction with a wide variety of ligands. Described in 1975 (Porath *et al.*, 1975), Lihme *et al.* (Lihme *et al.*, 1986) were who reported its application in affinity chromatography as an alternative to CNBr-activated gels. They coupled i) rabbit immunoglobulin for preparation of goat anti-rabbit immunoglobulin, ii) goat anti-rabbit immunoglobulin for preparation of rabbit immunoglobulin, iii) lectins and iv) L-fucose. Remarkably beads coupled to lectins or saccharides are currently used in glycomics (Kaji *et al.*, 2003; Bunkenborg *et al.*, 2004; Yang & Hancock, 2004). Vinyl sulfone activated agarose has been the bead of choice to study the interaction of pepsin with aromatic amino acids (Frydlova *et al.*, 2004; Frydlova *et al.*, 2008) or for the isolation of

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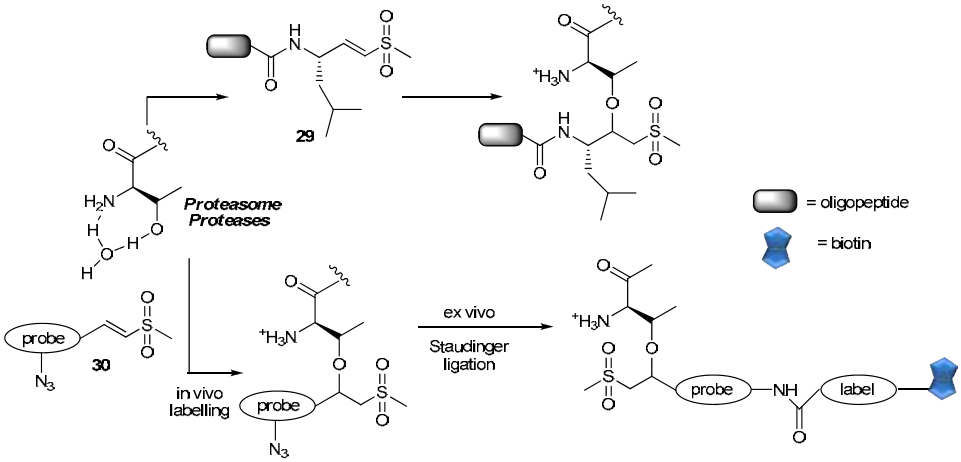

Fig. 8. Representative examples of vinyl sulfone-based ABPs targeting proteasomal proteases.

phophorylcholine-binding proteins (Liberda *et al.*, 2002a) by affinity chromatography. To our knowledge the only work using vinyl sulfone activated sepharose that may resemble Proteomics is that published by Liberda et al, (Liberda *et al.*, 2002b) who immobilized mannan to isolate mannan-binding bull seminal proteins that were identified by N-terminal amino acid sequencing.

In principle, the use of silica in Proteomics is discouraged since as predicted by Arai and Norde (Arai & Norde, 1990) macromolecules are adsorbed onto silica via strong electrostatic interactions and the secondary structure of the proteins can be distorted. However, the authors' group has reported the functionalization of silica with vinyl sulfone (**31**, Fig. 9) to yield a novel "ready to use" pre-activated material that reacts with biomolecules in mild conditions, preserves the activity of enzymes and can be used as an open support in Proteomics (Morales-Sanfrutos *et al.*, 2010b; Ortega-Munoz *et al.*, 2010). In a recent work (Traverso *et al.*, 2010), the application of this hybrid organic-inorganic material to Proteomics was further validated in a pull down experiment that demonstrated the different affinity of two pea h-type thioredoxins for proteins from a crude extract: thioredoxin h2 interacted with classical antioxidant proteins whereas thioredoxin h1 was able to capture a transcription factor, suggesting a regulatory role. These results support the use of vinyl sulfone silica in Proteomics for the study of protein-protein interactions.

3.4 Vinyl sulfone-based microarray technologies

Arrays are another important tool in Proteomics. They rely on the interaction between an immobilized probe and the molecules in the sample being analyzed. Immobilization is an important variable and different methods of both covalent and non-covalent immobilization are used with their pros and cons. Up to the present, a limited number of reports have described the preparation and use of different vinyl sulfone-modified surfaces in the construction of microarrays, the majority of them focused on potential applications in other omics (*vide infra* section 4). Only one of these contributions describes a gelatin-based substrate functionalized with vinyl sulfone groups for fabricating protein arrays (Fig. 10)

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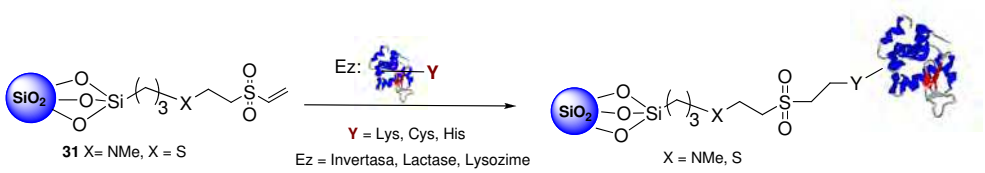

Fig. 9. Immobilization of enzymes onto vinyl sulfone silica

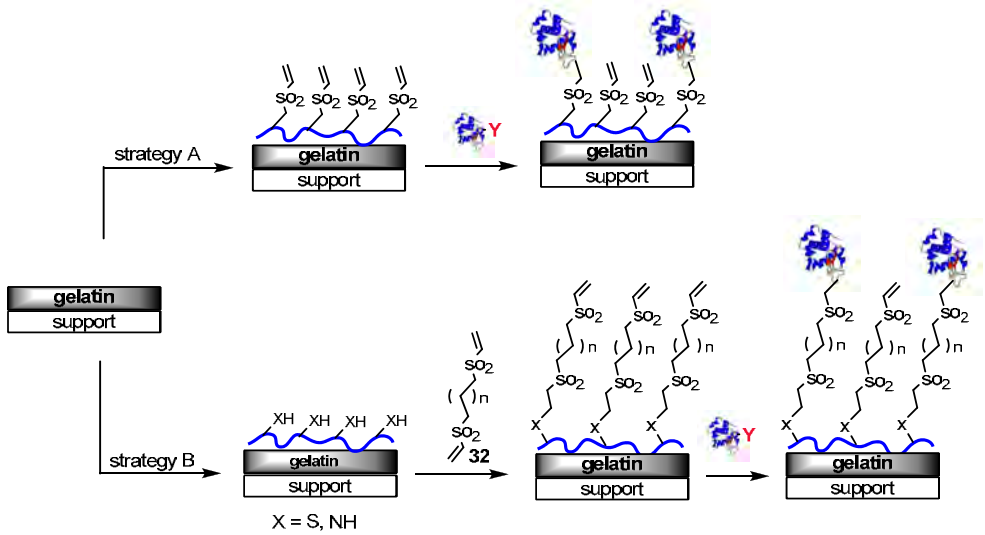

Fig. 10. Vinyl sulfone-gelatine protein microarrays

(Qiao *et al.*, 2003). The rationale behind the design of these materials are the use of gelatin coating to eliminate non-specific protein binding and the affixing to this gelatin surface of a vinyl sulfone derivatized polymer scaffold to enable them for the direct immobilization of proteins (strategy A). In an alternatively strategy, the gelatin surface is first affixed with a polymer scaffold rich in thiols or amine groups, then reacted with a bis(vinylsulfonyl) compound (**32**) and finally bonded to a protein capture agent such as an antibody (strategy B).

3.5 Vinyl sulfone-based post-translational modifications

Protein post-translational modifications increase the functional diversity of the proteome and the access to pure protein derivatives is essential in order to gain insight into structure-activity relationships and their biological role. Among the different post-translational modifications of proteins, glycosylation is the most prevalent one, occurring in at least 50% of all proteins (Apweiler *et al.*, 1999). However, the fact that glycosylation is not template driven makes the large scale production of glycoproteins a challenging task that has been approached by biological, enzymatic and chemical strategies (Davis, 2002; Bennett & Wong, 2007; Gamblin *et al.*, 2008a; Bernardes *et al.*, 2009). The authors' group has already demonstrated the feasibility of the vinyl sulfone functionalization of the anomeric carbon on

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different carbohydrates (**33**) as a procedure for the chemical glycosylation of proteins (Fig. 11) (Lopez-Jaramillo *et al.*, 2005) and current work is focused on its application in the context of glycoscience to explore protein-carbohydrate interactions. In this context, a model system comprising four monosaccharides (L-fucose, D-glucose, D-mannose and N-acetyl-D-glucosamine) and three disaccharides (lactose, maltose and melibiose) with a vinyl sulfone group at the anomeric carbon were reacted with four model proteins (lysozyme, BSA, concanavalin A and lumazine) (unpublished results). Enzyme-linked lectine assays (ELLA) of the resulting neoglycoconjugates with lectins revealed that the extent of binding of the lectins was consistent with their carbohydrate-binding specificity: Concanavalin A (ConA) showed binding with proteins derivatized with vinyl sulfone D-mannose while peanut agglutinin (PNA), ulex europeaus aggluttinin (UEA) and wheat germ agglutinin (WGA) interacted with those proteins reacted with vinyl sulfone derivatized lactose, L-fucose and N-acetyl-D-glucosamine, respectively.

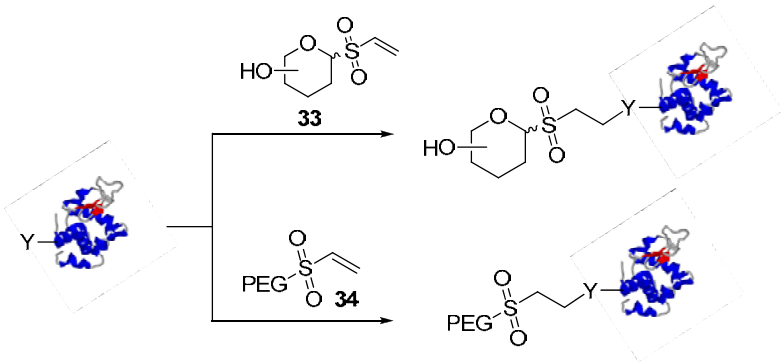

Fig. 11. Vinyl sulfone based glycosylation and PEGylation of proteins

Although *stricto sensu* PEGylation (covalent attachment of polyethylene glycol –PEG-chains) is not a post-translational modification, it is important for pharmaceutical and biological applications (Brannon-Peppas, 2000). Covalent attachment of PEG to proteins shields their antigenic and immunogenic epitopes, interferes with the receptor mediated uptake and prevents recognition and degradation by proteolytic enzymes. Vinyl sulfone chemistry has been exploited in this field. Poly(ethylene glycol) vinyl sulfone (**34**, Fig. 11) was synthesized and its highly selective reaction with thiol groups relative to amino groups at pH lower than 9 was described (Morpurgo *et al.*, 1996). The idea of using vinyl sulfone derivatives for PEGylation at cysteine residues is still accepted and later contributions reported the use of these methodology although being aware of the side reaction with lysine at elevated pH (Roberts *et al.*, 2002).

4. Vinyl sulfones in other omics sciences

The complete sequencing of the human genome has led to a new era referred to as omic sciences that comprise a wide range of disciplines aims at analyzing the relationships among the different elements of various omes. A common characteristic is the use of innovative technology platforms that allow the high-throughput detection and identification of the large amount and variety of molecules expressed in living organisms. Both immobilization

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on a solid surface either for affinity chromatography applications or as arrays and coupling to other biomolecules are important elements shared by all omic sciences.

In the context of immobilization, vinyl sulfone activated sepharose and vinyl sulfone silica are two open affinity chromatographic supports valid not only in Proteomics (*vide supra* section 3.4) but also in glycomics to isolate glycoproteins if lectins are immobilized or in genomics if amine or thiol functionalized oligonucleotides are used. In the particular case of glycomics, divinyl sulfone (DVS) has been used for the surface functionalization of either the wells of microtiter plates containing primary amino groups (Hatakeyama *et al.*, 1996; Hatakeyama *et al.*, 1997) or hydroxyl-terminated self-assembled monolayers (SAMs) on Au (Cheng *et al.*, 2011). Both materials have demonstrated their capability for the direct chemical immobilization of natural and chemically derived carbohydrates as well as glycoproteins and their applicability for the development of a simple assay to determine lectin activity, in case of the vinyl sulfone functionalized microplates, and for the fabrication of a glycan microarray, in case of the vinyl sulfone derivatized SAMs. On the other hand, the activation of molecules via vinyl sulfone functionalization is a wide scope strategy for labeling (colorants and fluorophores) and tagging (biotin) not limited to Proteomics. Finally, in the particular case of glycomics, vinyl sulfone derivatization of sugars is especially appealing since as described above (section 3.5) it is suitable for the synthesis of neoglycoconjugates that are recognized by lectins.

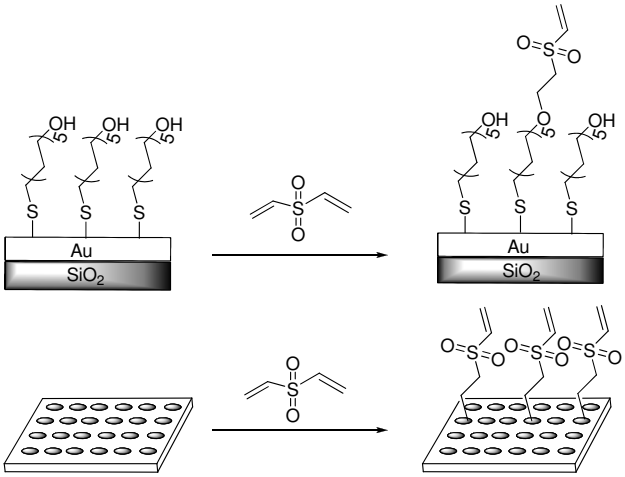

Fig. 12. Divinyl sulfone (DVS) functionalization of surfaces (SAM and microtiter plates) for applications in glycomics

In lipidomics, immobilized lipids are a valuable tool for the characterization and study of the lipid-protein interaction. This issue is not new in pharmaceutical industry where some of the most famous drugs target lipid-metabolizing enzymes. For example, atorvastatin (Lipitor from Pfizer) is a competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA), the rate controlling enzyme involve in the metabolic pathway of cholesterol, or Celecoxib (Celebrex from Pfizer) is a selective inhibitor of cyclooxygenase-2, enzyme responsible for the conversion of arachidonic acid into prostaglandin that is the molecule involved in inflammation and pain. Thus, lipid profiling for the identification of

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metabolic pathways and enzymes involved is an area of interest in lipidomics (Wenk, 2005). Both covalent and non-covalent immobilization strategies do not seem to compromise the activities of the group of phosphoinostides (Feng, 2005). In order to promote the covalent immobilization with surfaces, reactive groups including amine among others are introduced in the lipid molecule and in this context the above mentioned microarrays based in vinyl sulfone derivatized monolayers (SAMs) (Cheng *et al.*, 2011) can be applied to the synthesis of lipid microarrays. Another important issue is protein lipidization where vinyl sulfone chemistry can play a role. In general it is assumed that the hydrophobic acyl groups are involved in protein-membrane interaction and protein-protein interactions (McCabe & Berthiaume, 1999; Taniguchi, 1999). Historically, fatty acylation has been divided into two classes: cotranslational addition of myristate to N-terminal glycine through amide linkage (myristoylation) and post-translational addition of palmitate through a thioester linkage to cystein. Both N-terminal and thiol groups can be targeted by vinyl sulfone chemistry. Finally, it should be mentioned that, although for a different purpose, the authors' group has reported the synthesis of alkyl vinyl sulfones and vinyl sulfone functionalization of cholesterol and their reaction with poly(amidoamine) (PAMAM) dendrimers for the preparation of dendrimers-based nonviral gene delivery vectors with improved transfection efficiencies (Fig. 13) (Morales-Sanfrutos *et al.*, 2011).

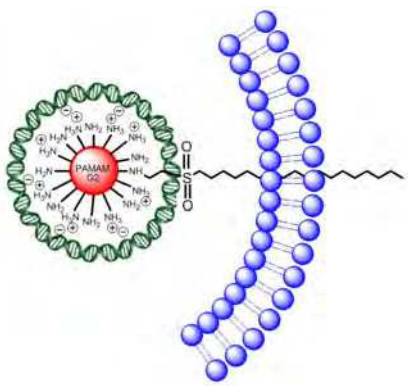

Fig. 13. Alkyl sulfonyl derivatized PAMAM-G2 dendrimers engineered by vinyl sulfone chemistry as nonviral gene delivery vectors with improved transfection efficiencies.

In the field of genomics, a method for gene analysis by simultaneously performing the polymerase chain reaction (PCR) reaction and the hybridization reaction of an oligonucleotide, a polynucleotide or a peptide nucleic acid fixed on a vinylsulfonyl functionalized silicate glass micro-array obtained by a tandem treatment with an amino silane coupling agent and a bis(vinylsulfonyl) compound has been reported (Iwaki *et al.*, 2004). This method avoids traditional operations where PCR and hybridization reactions are separately performed for gene analysis.

5. Conclusion

The reactivity of the vinyl sulfone function toward thiol and amine groups that are naturally present or routinely introduced in most of biomolecules makes it a wide scope strategy for

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functionalization with a clear potential in omic sciences. The examples in the previous sections are indicative to the usefulness of vinyl sulfone reactivity in Proteomics owed to their excellent capability to act as Michael acceptors in physiological conditions (aqueous media, slightly alkaline pH and room temperature) that preserves the biological function of the proteins with no formation of by-products. However, despite the existence of a body of knowledge in bibliography, the applications of vinyl sulfones are only partially exploited and the vast potential of these compounds for targeting biological macromolecules is yet to be unearthed. For the particular case of Proteomics it is important to recall the presence of a panoply of potential reactive groups in proteins and the dependence of their reactivity on the neighboring residues. Nevertheless, vinyl sulfone group is appealing despite the modification of a particular residue is far from trivial since this is not a critical issue for many applications in Proteomics. Its impact in other sciences is promising but still unexplored.

6. Acknowledgment

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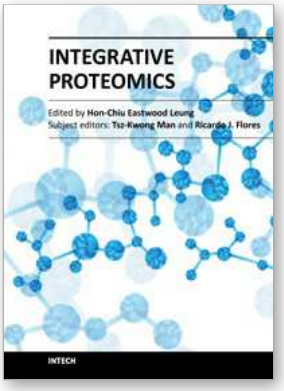

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Proteomics was thought to be a natural extension after the field of genomics has deposited significant amount of data. However, simply taking a straight verbatim approach to catalog all proteins in all tissues of different organisms is not viable. Researchers may need to focus on the perspectives of proteomics that are essential to the functional outcome of the cells. In Integrative Proteomics, expert researchers contribute both historical perspectives, new developments in sample preparation, gel-based and non-gel-based protein separation and identification using mass spectrometry. Substantial chapters are describing studies of the sub-proteomes such as phosphoproteome or glycoproteomes which are directly related to functional outcomes of the cells. Structural proteomics related to pharmaceutics development is also a perspective of the essence. Bioinformatics tools that can mine proteomics data and lead to pathway analyses become an integral part of proteomics. Integrative proteomics covers both look-backs and look-outs of proteomics. It is an ideal reference for students, new researchers, and experienced scientists who want to get an overview or insights into new development of the proteomics field.

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Phone: +385 (51) 770 447
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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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