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To Cleave or Not To Cleave in XL-MS?

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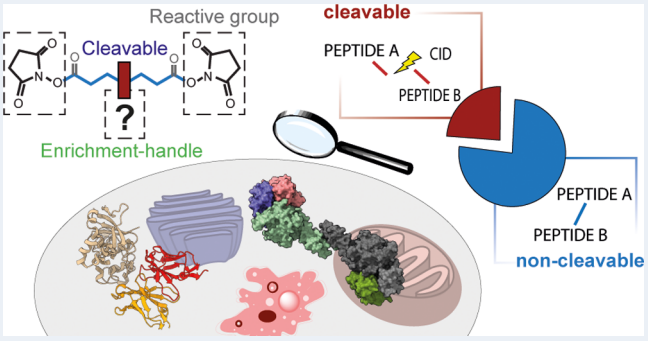
Supporting Information

ABSTRACT: Cross-linking mass spectrometry (XL-MS) is an efficient technique for uncovering structural features and interactions of the in-solution state of the proteins under investigation. Distance constraints obtained by this technique are highly complementary to classical structural biology approaches like X-ray crystallography and cryo-EM and have successfully been leveraged to shed light on protein structures of increasing size and complexity. To accomplish this, small reagents are used that typically incorporate two amine reactive moieties connected by a spacer arm and that can be applied in solution to protein structures of any size. Over the years, many reagents initially developed for different applications were adopted, and others were specifically developed for XL-MS. This has resulted in a vast array of options, making it difficult to make the right choice for specific experiments. Here, we delve into the previous decade of published XL-MS literature to uncover which workflows have been predominantly applied. We focus on application papers as these represent proof that biologically valid results can be extracted. This ignores some more recent approaches that did not have sufficient time to become more widely applied, for which we supply a separate discussion. From our selection, we extract information on the types of samples, cross-linking reagent, prefractionation, instruments, and data analysis, to highlight widely used workflows. All of the results are summarized in an easy-to-use flow chart defined by selection points resulting from our analysis. Although potentially biased by our own experiences, we expect this overview to be useful for novices stepping into this rapidly expanding field.



INTRODUCTION

Structural biology aims at uncovering structural information from proteins and protein complexes. The resulting structural details of the investigated molecular machines provide crucial insights that extend our understanding of their function in complex biological systems. This might ultimately help to develop treatments for diseases for which structural deviations in and/or interactions between proteins are causative. Classically, this field has been dominated by techniques such as X-ray crystallography, nuclear magnetic resonance (NMR), and electron microscopy (EM). Notably, the latter has in recent years seen a sharp increase in applications to samples in a frozen hydrated state after vitrification (cryo-EM).[1] Mass spectrometry (MS) based proteomics in its many guises has also come to prominence in the past decade as a complementary technique to these established approaches, providing structural details of the studied proteins either in-solution or in the gas phase.[2] Techniques like top-down and native MS,[3] hydrogen/deuterium exchange (HDX) MS,[4] covalent labeling[5] and cross-linking mass spectrometry (XL-MS) have been used to supplement details to the extracted structural information. Each approach uniquely provides insights at the level of protein sequences, protein stoichiometry, interaction interfaces, and post-translational modifications (PTMs) involved in protein function. Combined, this provides a toolbox to the structural biologists that is helpful to uncover details of protein structures that are not always accessible in atomic detail with classical structural determination approaches. In the wider mass spectrometry toolbox, XL-MS is *the* technique that provides spatial distance constraints representative of the in-solution state of intact proteins or between interacting intact proteins. It leverages small reagents, typically incorporating two amine reactive moieties capable of forging a covalent bond between amino acids in close proximity. After proteolytic digestion, the resulting products consist of unmodified peptides, peptides with the linker attached that is quenched on the other reactive end termed "monolinks", and peptide pairs covalently linked by the reagent termed "cross-links". The cross-linked peptides are structurally the most informative, although also monolinks carry

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information reflecting surface-accessible regions of proteins.[6] When the two cross-linked peptides originate from the same protein, termed intra-link, they can carry information on the folding and conformation of the protein; when the two peptides originate from two different proteins, termed inter-link, they carry information on the interaction interface between the two proteins. Cross-linking data is typically recorded using standard shotgun proteomics mass spectrometry workflows. Briefly, first an overview scan (FULL scan) provides insight into all available ionized molecules complete with their isotope information expressed by m/z and charge state, typically termed precursor ions. For those precursors interesting to investigate further, the isotope can be mass selected and fragmented by various peptide gas-phase fragmentation approaches. The resulting fragmentation spectra yield insight into the amino acid sequences of the peptides in a single MS^2 or multiple MS^n fragmentation scan(s).

Over the years, many reagents have been proposed for use in XL-MS experiments. These have, for example, been adopted from earlier work on bio-conjugation experiments, with the earliest mention of utilizing them to reveal protein structural information already in the 1970s.[7−9] In the intermediate period between those early experiments and the time-frame discussed in this review, a large amount of development work was done. For example, new mass spectrometric data acquisition routines, data analysis software, and applications to biologically relevant systems were performed. Over the previous decades, many reagents have been developed specifically for XL-MS applications, incorporating special features intended to improve the detection of cross-linked peptides by MS. This has resulted into five distinct design classes. The first class incorporates moieties intended to specifically enrich for cross-linked peptides, which is needed as the reaction efficiency of the available cross-linking reagents is low at an estimated 1−5%.[10,11] This leads to a situation where less than 0.1% of the sample actually consists of the desired cross-linked peptides. Typically, biotin is the enrichment handle, either by direct incorporation onto or by functionalization of the cross-linking reagent after the cross-linking reaction through 1,3 dipolar cycloadditions (click-chemistry).[12] However, as biotin proves difficult to elute due to its high affinity binding to streptavidin or avidin and might lead to steric hindrance due to its bulky nature, other enrichment handles have been proposed. Recently, incorporation of a phosphonic acid directly onto the cross-linking reagent has enabled IMAC enrichment with a handle of limited size and hence limited steric hindrance.[13] The second class incorporates a labile moiety that can be cleaved in the gas phase by collisional (CID/HCD) or electron transfer (ETD/ECD) fragmentation at preferably lower or comparable potentials than those where the amide backbone starts to fragment. Cleavable cross-linking reagents ease the identification process of the individual peptides of a peptide pair. In comparison, for so-called noncleavable cross-linking reagents the identification starts from a single precursor mass that is a combination of two unknown peptides. This leads to a combinatorial "explosion" of possible solutions, as every theoretically possible peptide needs to be combined with every other to find the right combination. Cleavable reagents preclude this "explosion" by exposing the masses of the individual peptides with parts of the cross-linker attached. Labile moieties have proven to be very efficient (e.g., sulfoxide moieties,[14] urea moieties[15] and the labile peptide bond between certain amino acids such as aspartic acid and proline[16]). However, software solutions have been proposed that ease the identification process for noncleavable cross-linking reagents. These algorithms utilize information present in the fragmentation spectrum to drastically limit the number of peptide combinations required to be made, like open searches[17] and use of sequence tags.[18] The third class incorporates heavy isotope labels in the cross-linking reagent. Mixing two aliquots cross-linked in parallel, one with a light reagent and one with a heavy reagent, provides distinct FULL and/or MS^n mass patterns that indicate whether a precursor is a peptide pair cross-linked with both the heavy and the light reagent. This ultimately eases the identification process, as many fragmentation scans can be ignored.[19] However, also here different approaches have been proposed utilizing specific diagnostic ions in the fragmentation spectra to make the same distinction.[20,21] Fortuitously, the isotope labels also enable relative quantitation of differentially treated aliquots to detect conformational changes in the proteins and/or protein complexes under investigation.[22] Recently, however, the use of N-terminal chemical labeling for quantitation in combination with cross-linking has been introduced, which circumvents the need for isotope coding directly in the cross-linking reagents.[23,24] The fourth class utilizes different spacer lengths to bridge smaller or larger distances. The smallest spacer length constitutes the so-called "zero-length" reagents[25,26] that directly link two amino acids in close proximity, which theoretically delivers the shortest length and consequently the best resolution. Spacers up to a length of 45 Å have also been proposed,[27] but most commonly a spacer distance around 11−12 Å is used. The fifth and last class uses alternative reactive groups to the most widely used NHS esters that are highly reactive for lysines. Targeting amino acid side chains different from lysines, such as cysteines, acidic residues, and non-specific targeting of amino acids by photoreactive groups can be beneficial.[9,28] These have, however, so far not been widely applied in more complex biological applications.

All these design classes were also mixed-and-mashed into new designs, resulting in a forest of available cross-linking reagents from which it can be difficult to select the most appropriate for a given experimental setup. By our count, 42 different homo-bifunctional noncleavable cross-linking reagents alone are available from a single vendor (Thermo Fisher, Rockford, IL), and many more vendors supply these and different reagents as well. To ease the onerous task of selecting the right reagent, we have mined a decade's worth of high-quality XL-MS literature to find common patterns for different types of experiments. As starting point for available reagents, we used the highly detailed reviews of the groups of Borchers[29] and Martinez[30] and mined the literature for their usage. Our final set of applications comprises 296 peer-reviewed papers (Supplementary Table 1), selected based on whether they either introduce the reagent for the first time or actually apply the reagent to address particular biological questions; i.e., review and technical development papers were excluded. This filter is applied, as we believe an actual application constitutes better proof of the usefulness of the reagent then a proof supplied by, e.g., a method development paper. In one instance, old data was re-analyzed to extract more information.[31] As this cannot be considered a new application, this publication was removed from the overall statistics.

From this set of papers alone, we uncover 55 different cross-linking reagents, highlighting the difficult task of selecting the

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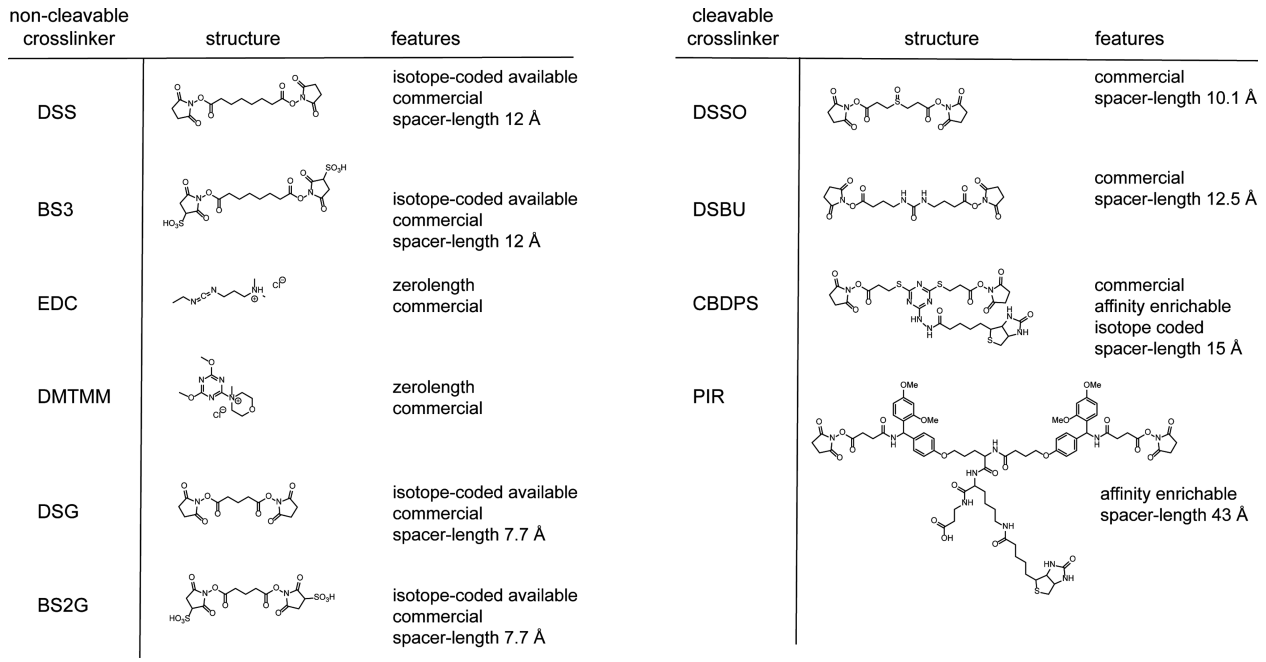

Figure 1. Overview of cross-linking reagents applied to biological applications.

right reagent. We can of course not completely rule out that papers were missed in our search, but from such a large set we believe the conclusions can nonetheless be generalized. To illustrate the results, we supply the structures and features of the most widely applied noncleavable and cleavable cross-linking reagents in Figure 1. To ease the final process of selecting the appropriate reagent and related experimental setup, we provide a flowchart, which step-by-step makes selection of one or more workflows straightforward.

A BIRD'S EYE VIEW ON XL-MS APPLICATIONS

Thanks to earlier pioneering work that for the first time combined chemical crosslinking with mass spectrometry, XL-MS is nowadays a popular method for the study of proteins, protein structures, and protein−protein interactions.[32−36] Challenges in XL-MS, such as the difficulties in detection of cross-linked products had been tackled not only by pure developments in mass spectrometric instrumentation, but also by innovative new cross-linker concepts such as isotope-coded and cleavable cross-linkers.[37] Over the years, many protein systems have been tackled, ranging from highly purified to complex lysates in all areas of cellular biology; for an overview see several reviews.[35,38] From the selected application papers it is clear that the field has gained traction between 2009 and 2016 and has now stabilized on approximately 40 application papers a year (Figure 2a). In this period, three major developments occurred that have likely driven this trend. The first was the development of database search engines specifically designed for XL-MS data that also became wider available for use in the community. Packages like xQuest, pLink, StravoX, and Xi were supplied to nonexpert laboratories who used XL-MS in combination with other structural biology techniques to unravel the structure of protein complexes either purified, like the ribosome,[39] proteasome,[40] RNA polymerase complexes,[41,42] or within their native environment, like the nuclear pore complex.[43] The second were the advances made in LC−MS/MS platforms, where new mass spectrometers started to offer the throughput and sensitivity to reliably identify the cross-linked peptide pairs present in the sample.[44] The third, and arguably most significant advance, was the "resolution revolution" in 2014 that vastly improved the information content of cryo-EM experiments.[45] XL-MS proved to be a perfect companion for cryo-EM, as it provides highly complementary information helping researchers to cope with lower resolutions in EM maps. To illustrate, more than half of the maps released in 2019 are still above 4 Å resolution (https://www.ebi.ac.uk/pdbe/emdb/statistics_num_res.html/), a resolution where additional structural information appears to still be advantageous. XL-MS provides insights into which proteins are present and how they are arranged inside an EM density. This has resulted in a very noticeable increase of applications from 2013 to 2015. In the same histogram, we subdivided the application papers into sample complexity categories for which a coarse division was made: low (one to two proteins), medium (purified complexes of three proteins and upwards), and high (proteome-wide samples like organelles and cells, intact, or lysed). From this subdivision, it is clear that the field is mostly focused on purified complexes, which fits well with combined applications with cryo-EM. Since 2014 more applications to high-complexity samples have been made; for now, these remain a rather small portion of the total applications.

Differentiating between gas-phase cleavable and noncleavable reagents, it is clear that the use of noncleavable cross-linking reagents outstrips the use of cleavable cross-linking reagents by some margin with ∼77% of applied cross-linking reagents being noncleavable (Figure 2b). This is not surprising, as these reagents have been available the longest and experimentalists are comfortable in applying them to protein structures based on experiences in, e.g., cryo-EM. This preference was also reflected in the recent community-wide comparative study (Figure 2a) in which solely expert laboratories were involved.[46] To understand whether cleavable cross-linking reagents are lagging in terms of applications because of later introduction, we plotted the application trend over time (Figure 2c). For the noncleavable reagents, it is clear

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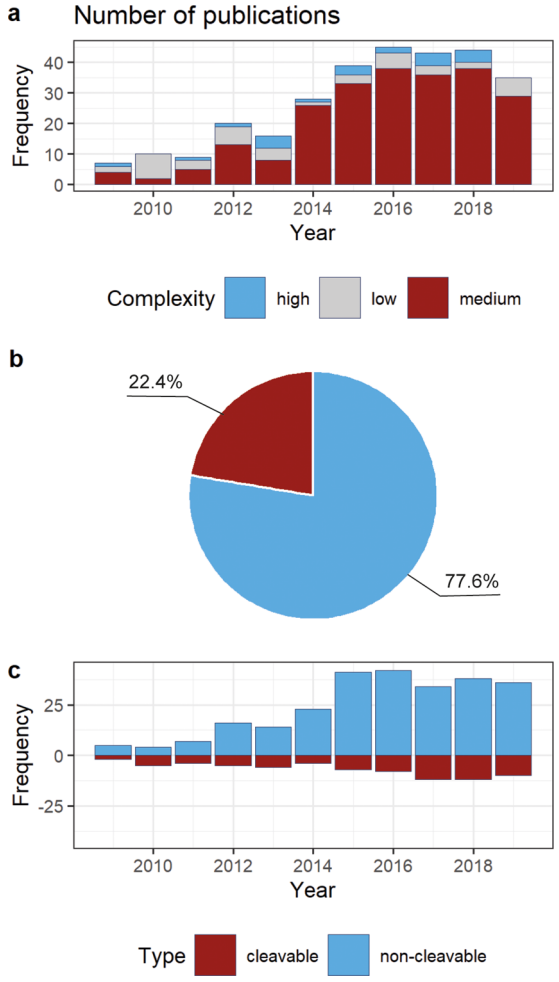

Figure 2. Evolution of the field of XL-MS. (a) Increase in XL-MS application papers over the last decade. (b) Applications broken down into those using cleavable vs noncleavable cross-linking reagents, provided separately as several publications utilize multiple cross-linking reagents. (c) Application trend of cleavable versus non-cleavable cross-linking reagents over the last decade.

their use has started to level after the initial explosion up until 2016, although they remain highly popular. The MS cleavable cross-linking reagents have started to gain traction in 2017. As the first version of this class was already introduced in 2005,[37] this appears to indicate that a lag phase from conception to wide-scale adoption can be expected for novel reagents. The introduction was followed by the release of data analysis solutions like XlinkX[47] and MeroX[48] capable of using the data generated by the gas-phase cleavable moiety of these reagents. In terms of number of applications, the cleavable type of linkers still has some way to go to get on par with the noncleavable linkers.

PREFRACTIONATION OF CROSS-LINKED PRODUCTS

As mentioned in the Introduction, the cross-linking reaction has a very low efficiency, leading to a maximum of 1−5% of residues in close proximity covalently linked.[10,11] To counter this, even for purified multiprotein complexes of relatively few subunits, the cross-linked products are typically prefractionated, leading to extensive and expensive measurement times. Separation of cross-linked products was performed in ∼88% of the applications considered, either at the protein level, peptide level, or both, depending on the sample complexity. Separation of cross-linked proteins by gel electrophoresis, either denaturing or native, and subsequent in-gel digestion still represents the most widely used methods and are applied in ∼28% of the cases considered, but only to low- to medium-complexity samples cross-linked with noncleavable reagents. This approach is particularly cost-effective as it can be performed without particular HPLC fractionation equipment and the cross-linked products can easily be visualized and excised from the gel. In-gel digestion, however, has a lower efficiency than in-solution digestion, and notably, in-gel digestion was in most cases not applied to medium- to high-complexity samples. The preference is mainly toward the enrichment of cross-linked peptides, which was applied in more than half of the studies. Peptide fractionation is especially beneficial for medium- to high-complexity samples, as it lessens the unwanted background of normal peptides and ultimately improves the spectral quality and facilitates downstream data analysis. The peptide mixtures can be fractionated using several approaches, for which the most used are size-exclusion chromatography (SEC), separating cross-linked peptides based on their higher mass with respect to normal peptides, and strong-cation exchange (SCX), which relies on the fact that cross-linked peptides carry higher amounts of charges. For medium- to high-complexity samples, SEC-HPLC is preferentially used (∼66%), followed by SCX-HPLC (∼11%). These fractionation techniques are used for noncleavable as well as cleavable cross-linking reagents. For noncleavable cross-linking of low- to medium-complexity samples, a simpler method is preferred with in-house packed stage-tips applied in over 80% of the cases where SCX is the selected peptide fractionation method. This choice can potentially also be driven by the sample amounts available, for which the stage-tips can handle much lower amounts. An alternative approach, which has been rarely applied so far (<9%), is the use of cross-linking reagents that carry an affinity tag and can therefore be separated from the noninformative peptides by affinity purification.

NON GAS-PHASE CLEAVABLE CROSS-LINKING REAGENTS

Since their initial application, almost four decades ago,[49] DSS and BS3 have been used in an increasingly wide range of applications in proteomic research. DSS and BS3 are essentially almost identical reagents, differing only in the amine-reactive group used to target lysine residues (Figure 1). DSS contains a classical NHS ester, whereas BS3 contains a water-soluble sulfo-NHS ester. Both reagents represent an efficient and cost-effective means to covalently cross-link lysines in proteins and can readily be combined with mass spectrometry. As such they represent an almost ideal reagent for XL-MS applications, which is evident from the fact that combined they are by far the most used cross-linking reagents, either alone or in combination with other reagents, at ∼59% for the last decade (Figure 3b). This means that these reagents can be considered as the driving force for wider acceptance of XL-MS within structural biology studies. Even though relative to their counterparts they appear to be the most reactive reagents, these reagents also offer a low likelihood of forming a covalent bond between amino acids in close proximity. This notwithstanding, their wide application is noteworthy with many high-impact applications in the last decade. This appears mostly driven by the combination with cryo-EM, with ∼76% of

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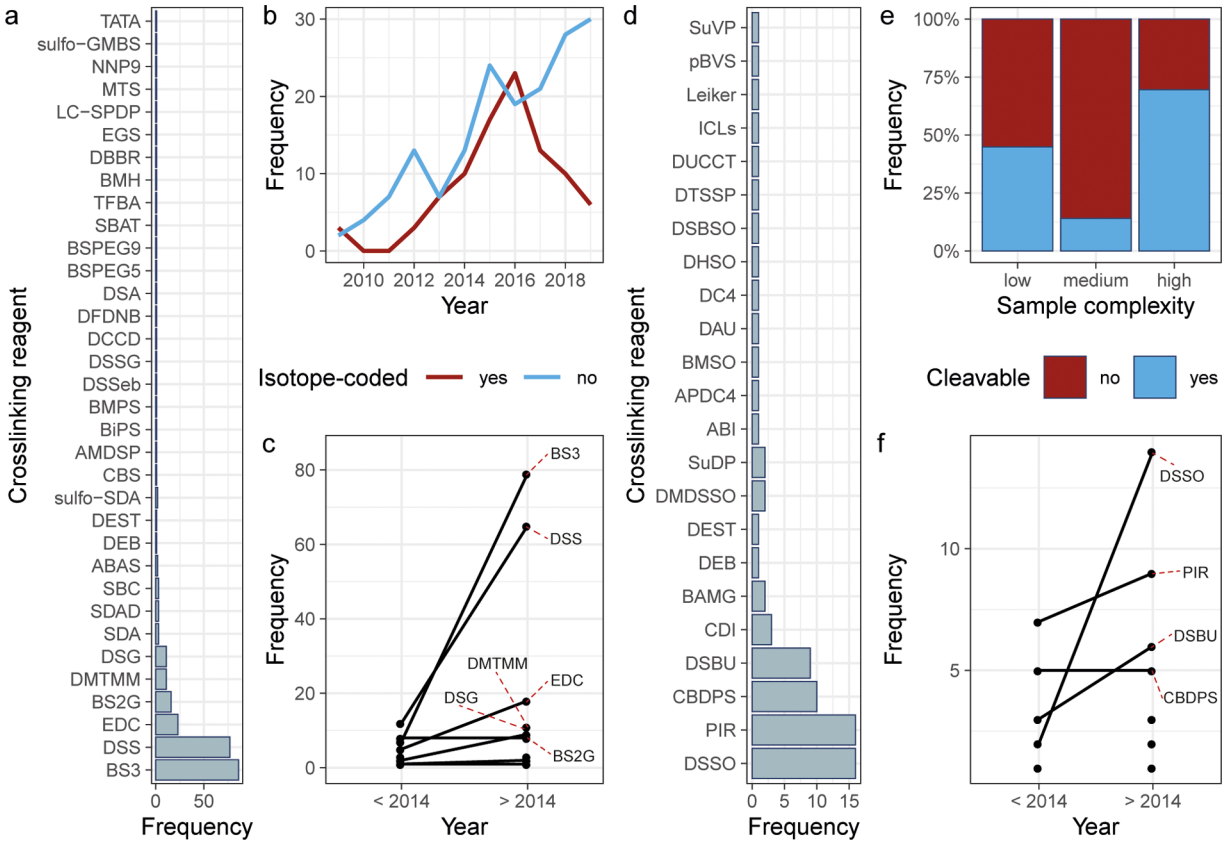

Figure 3. Statistical analysis of the application of cross-linking reagents. (a) Frequency of use for noncleavable cross-linking reagents. (b) Use of isotope coding in the reagents over time. (c) Growth of utilization for specific noncleavable reagents before and after 2014. (d) Frequency of use for noncleavable cross-linking reagents. (e) Sample complexity grouped for cleavable and noncleavable reagents. (f) Growth of utilization for specific cleavable reagents before and after 2014.

the studies using noncleavable cross-linking reagents utilizing integrative structural approach for which cryo-EM represents more than half. To ease the identification of fragmentation spectra containing cross-linked peptides, stable isotope strategies were employed, as described in the Introduction. Their use, however, has diminished since 2016 (Figure 3b). Possible reasons for this include: (I) the frequently used hydrogen and deuterium versions can result in varying retention times, complicating identification during data analysis, (II) the isotope-coded versions are relatively expensive and (III) by mixing light and heavy isotope-coded cross-linked samples, the intensity of cross-linked precursor ions is decreased hampering identification of low abundant cross-links. With ongoing advances in high resolution mass spectrometers and insights into diagnostic ions also providing signatures specific to the reagent, the benefit of using isotope labels likely has proven to be too costly.

Next to BS3 and DSS, variants in spacer length of these reagents are also widely used. Most applied of these variants are DSG and BS2G, comprising a shorter spacer length of 7.7 Å, and which are also available in isotopically labeled forms. Aside from the massive growth in applications of DSS and BS3 and their variants, a notable increase of usage was observed for the so-called zero-length cross-linking reagents, like EDC and DMTMM (Figure 3c). These are standard reagents in organic synthesis used to form amide bonds between amines and carboxylic acids and these reagents have excellent water solubility. Both reagents act as activating reagents to form an activated ester leaving group on the acidic side chains of aspartic and glutamic acids. After this step, amines from the lysine can attack to form a stable amide bond. EDC and DMTMM act as the condensation reagents between acidic residues and the side chains of lysines, resulting in a "zero-length" covalent bond. Whereas DMTMM reacts most optimally at neutral pH, EDC is more effective at lower pH (pH 4−6.5) as the reagent is only functional in its protonated state. EDC is often used in combination with NHS or sulfo-NHS to in situ form an NHS-ester on the acidic amino acid side chains, but this addition is, however, not necessarily required. Since both lysines as well as acidic side chains are targeted, additional or complementary information to lysine-only cross-linking reagents is likely obtained. Another potential benefit of these reagents is in the study of tight interactions and tightly packed domains in proteins with coiled−coil structural motifs or in proteins with lower lysine content. Both reagents are commercially available at low cost, are highly effective and can be analyzed with the same data analysis strategies as used for the analysis of protein samples cross-linked with noncleavable NHS-ester cross-linking reagents.

Steadily, but less applied, are hetero-bifunctional non-cleavable cross-linking reagents such as SDAD, comprising a lysine-reactive NHS-ester in combination with a photoreactive diazirine group that is unspecific in targeting amino acids. The use of photoreactive groups on cross-linking reagents appears beneficial for obtaining distance constraints that differ from those obtained by homo-bifunctional NHS-ester reagents.[28,50] Their promiscuous and unspecific nature in terms of targeted amino acids, however, complicates the data analysis by

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crowding the fragmentation spectra as the linker can be attached at many positions simultaneously. This is likely also the reason that no homo-bifunctional photoreactive cross-linking reagents currently exist. However, the combination of a lysine-reactive group with one photoreactive groups is feasible for data analysis, although this relatively young class of reagents was mostly applied so far to low complexity samples with a focus on method development. New software developments and search algorithms may likely increase their usage in the near future.

GAS-PHASE CLEAVABLE CROSS-LINKING REAGENTS

The complexity of interpreting the data of noncleavable reagents has driven much effort into the development of gas-phase cleavable cross-linking reagents.[51] These have the advantage of liberating the cross-linked peptides from their covalent bond in the gas phase, allowing for interrogation of each peptide individually. Therefore, highly complex mixtures such as whole cell lysates,[52] cellular compartments,[53,54] and tissues[55] could successfully be investigated by XL-MS. Of the available cleavable cross-linking reagents, DSSO,[14] PIR,[27] CBDPS,[56] and DSBU[15] are the most applied reagents (Figure 3d). Breaking up the applications on sample complexity and gas-phase cleavability shows that cleavable reagents are predominantly used for high-complexity samples (e.g., cell lysates; Figure 3e), usually in combination with prefractionation of cross-linked peptides. Surprisingly, these reagents are also applied often to low complexity samples (i.e., one or two proteins); this can, however, be attributed to the inclusion of papers initially describing the linker, which are tested first on low complexity protein standards. When these are excluded, the percentage of application of cleavable cross-linking reagents to low complexity samples is lower than 18%. Noncleavable cross-linking reagents are, in fact, predominantly applied to medium-complexity samples (i.e., purified complexes), although some first examples have emerged of application of these reagents to high complexity samples.[57] By far the largest growth in applications can be observed for the commercially available lysine-reactive reagent DSSO, which incorporates a sulfoxide functionality as cleavable moiety. Upon low CID- or HCD-fragmentation energy, the sulfoxide eliminates to form an alkene and a sulfenic acid fragment, separating the cross-linked peptides into two linear peptides. Its spacer length and size are very comparable to the popular noncleavable reagents BS3 and DSS. Next to this reagent, a commonly applied cleavable reagent is DSBU, which incorporates a urea moiety as labile functionality.

The second most applied are the PIR cross-linking reagents. PIR stands for "Protein Interaction Reporter" and is a conceptual term for cross-linking reagents using two incorporated labile groups that liberate the two individual peptides after cleavage as well as a diagnostic reporter ion (Figure 1). The labile bonds can be cleaved in the mass spectrometer by CID, ECD, or IRMPD or can be cleaved with photoactivation upon electrospray ionization. Early versions incorporated as cleavable moiety a "Rink structure", which is known as a structural motif on the connection site between the solid phase material and the growing peptide chain during peptide synthesis using the Boc-strategy. A more recent version, BDP-NHP,[58] incorporates a labile D−P peptide bond. Clearly, the design of PIR reagents is inspired by solid-phase peptide synthesis, which makes the concept modular. PIR reagents, however, span a relatively large spacer length of up to 45 Å. This potentially makes these reagents more applicable to interaction studies than to structural studies of protein complexes due to steric hindrance. One of the main advantages of using PIR reagents is that cross-linked peptide pairs can be enriched by affinity purification (AP; avidin, streptavidin binding). This enrichment approach has been used either alone or after SCX-HPLC separation, with the latter approach being particularly beneficial for high complexity samples. The third most applied cleavable cross-linking reagent is CBDPS,[56] which incorporates a labile C−S bond distal to the cyanuric group as cleavage site. CBDPS unifies multiple features for the cross-linked peptide identification such as isotopic labeling and a biotin for avidin enrichment. This reagent is also commercially available.

As mentioned previously, compared to noncleavable reagents the cleavable options have been applied far less. We hypothesize this is due to the need for high-end mass spectrometry equipment, which offer the potential to perform extra fragmentation events on fragments produced in a first round of fragmentation (MS^n scans). This is likely no longer a consideration, as recently it was demonstrated that with simpler instrumentation good results can also be achieved.[59] Of note here is however that data analysis software like pLink now also offer the option to search data of highly complex mixtures cross-linked with noncleavable reagents.[60] Potentially, this will somewhat hamper the adoption of gas-phase cleavable reagents in favor of the noncleavable cross-linking reagents as these software options improve further.

AMINO ACID REACTIVE CHEMISTRY

A large amount of emphasis has in recent years been placed on the development of cross-linking reagents offering different amino acid reactive chemistries. For example, reagents targeting acidic residues, cysteine residues, and photo reactive moieties have been developed. This may suggest there are biological questions that can be better answered by these reactivities. However, upon inspection of the published applications by far the most utilized reactivity is lysine-to-lysine (K−K) cross-linking (Figure 4). This is not entirely surprising given the wide application of DSS and BS3 that both use NHS chemistry to target lysines. Further inspection reveals that the second most applied are the zero-length cross-linking reagents (EDC and DMTMM), which provide an orthogonal approach targeting lysines as well as acidic residues. The zero-length cross-linking reagents EDC and DMTMM are also used in combination with dihydrazine reagents to covalently

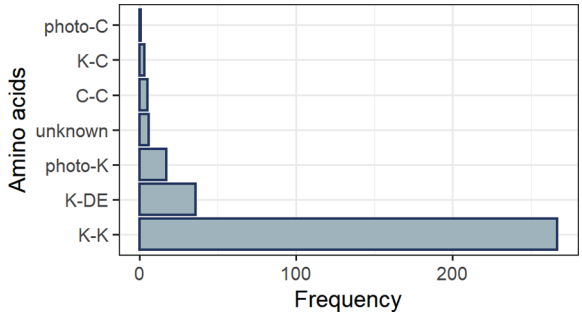

Figure 4. Statistical analysis on the frequency of use for cross-linking reagents targeting specific amino acid pairs.

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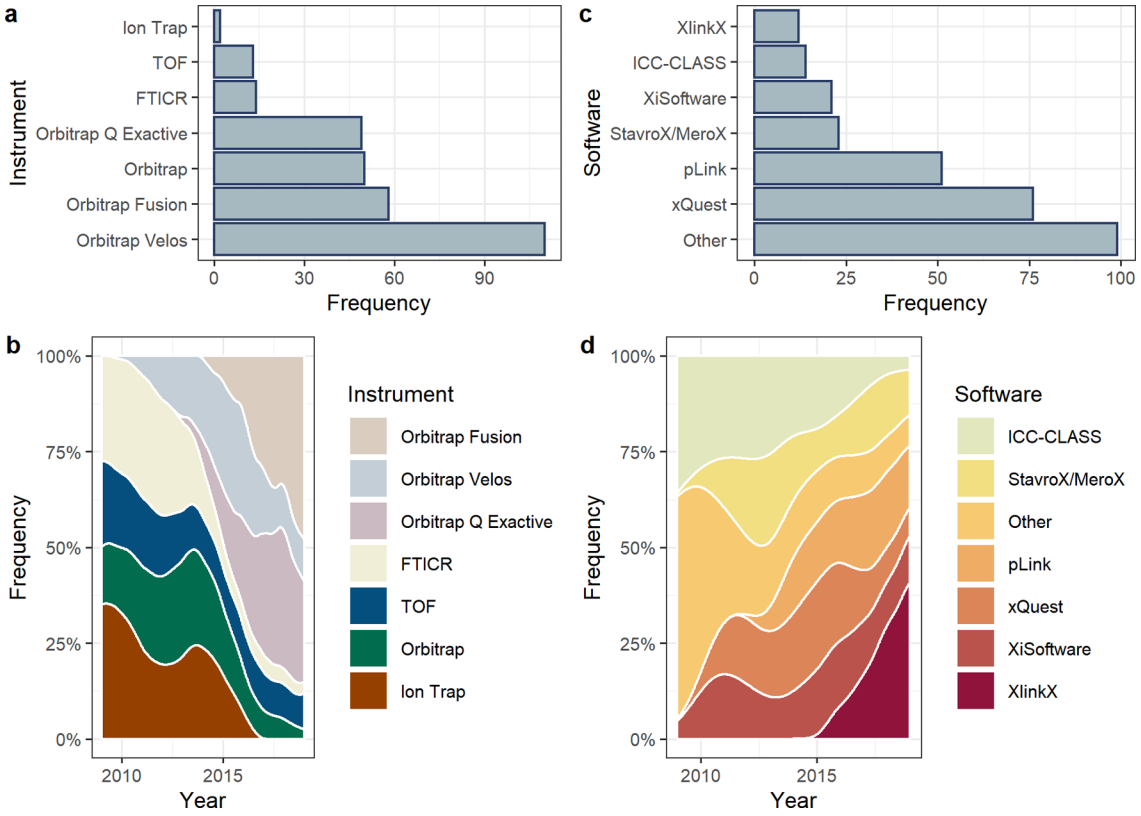

Figure 5. XL-MS analysis trends over time. (a) Used mass spectrometry platforms. (b) Mass spectrometry platforms usage plotted versus time. (c) Used data analysis pipelines. (d) Data analysis pipelines usage plotted versus time.

connect activated acidic residues (DE-DE);[10] however, for visualization purposes and due to the fact that acidic cross-linking with dihydrazines is rarely applied, we combined in the current analysis acidic and zero-length cross-linking. The remaining chemistries, including cysteine-to-cysteine (C−C) and lysine-to-any residue with photoactivatable reactive groups (photo-K), have apart from the reagent genesis papers seen little to no application. Notwithstanding, their introduction is relatively recent and these reagents can still find application to biological questions.

WORKFLOWS IN XL-MS

The selected set of papers also provides an excellent resource for detecting trends in data acquisition and analysis. To investigate data acquisition options, the different instruments used in the published studies were extracted from the papers. To enhance the overview, similar platforms received the same label (e.g., Orbitrap Velos and Elite represent a very similar platform and both received the same label) resulting in seven platforms, dominated by the Orbitrap Velos (Figure 5a). Up until 2014, relatively few applications were published, and the landscape is dominated by TOF, FTICR, and ion trap platforms, a rather heterogeneous mixture of platforms. After 2014, the landscape became almost completely dominated by the most advanced Orbitrap platform at each timepoint (Figure 5b). These platforms were sufficiently sensitive to support the resolution revolution inspired explosion of XL-MS studies and are updated periodically to the latest advances in mass spectrometry technology. This suggests that more sensitivity is still a top priority for these types of studies.

To investigate data analysis options, the different software solutions used in the published studies were also extracted from the papers. To enhance the overview, similar data analysis solutions received the same label (e.g., xQuest and xQuest combined with xProphet received the label xQuest) and rarely used options received the label "other" resulting in seven solutions. The list is dominated by xQuest[61] and pLink,[60] data analysis options that have been around the longest (Figure 5c). New software has become available as well, mostly with an initial focus on cleavable strategies like Merox[48] and XlinkX[47] but also with a focus on noncleavable strategies like Xi.[62] Over time, the major software solutions have, however, started to receive equal usage (Figure 5d). Each of these has specific solutions built to support their associated data acquisition methodologies and should likely best be utilized with their associated protocols.

CONCLUSIONS AND OUTLOOK

By reviewing the XL-MS application literature produced in the preceding decade, interesting insights into the trends pervading the field can be gained. From our analysis, by far the most widely applied approach constitutes the use of the non-cleavable cross-linking reagents DSS and BS3 in conjunction with data acquisition platform Orbitrap Velos/Elite and data analysis software xQuest and pLink. Of note is that for both the data acquisition as well as the data analysis options the field appears to be in flux. For the data acquisition platforms, the field is tracking the ongoing developments in mass spectrometry platforms with each ongoing year witnessing increasing amounts of applications for the most advanced platforms. We predict that this trend will simply continue as especially the specialized mass-spectrometry oriented laboratories keep investing in the most advanced data acquisition platforms. For the software the picture is more convoluted,

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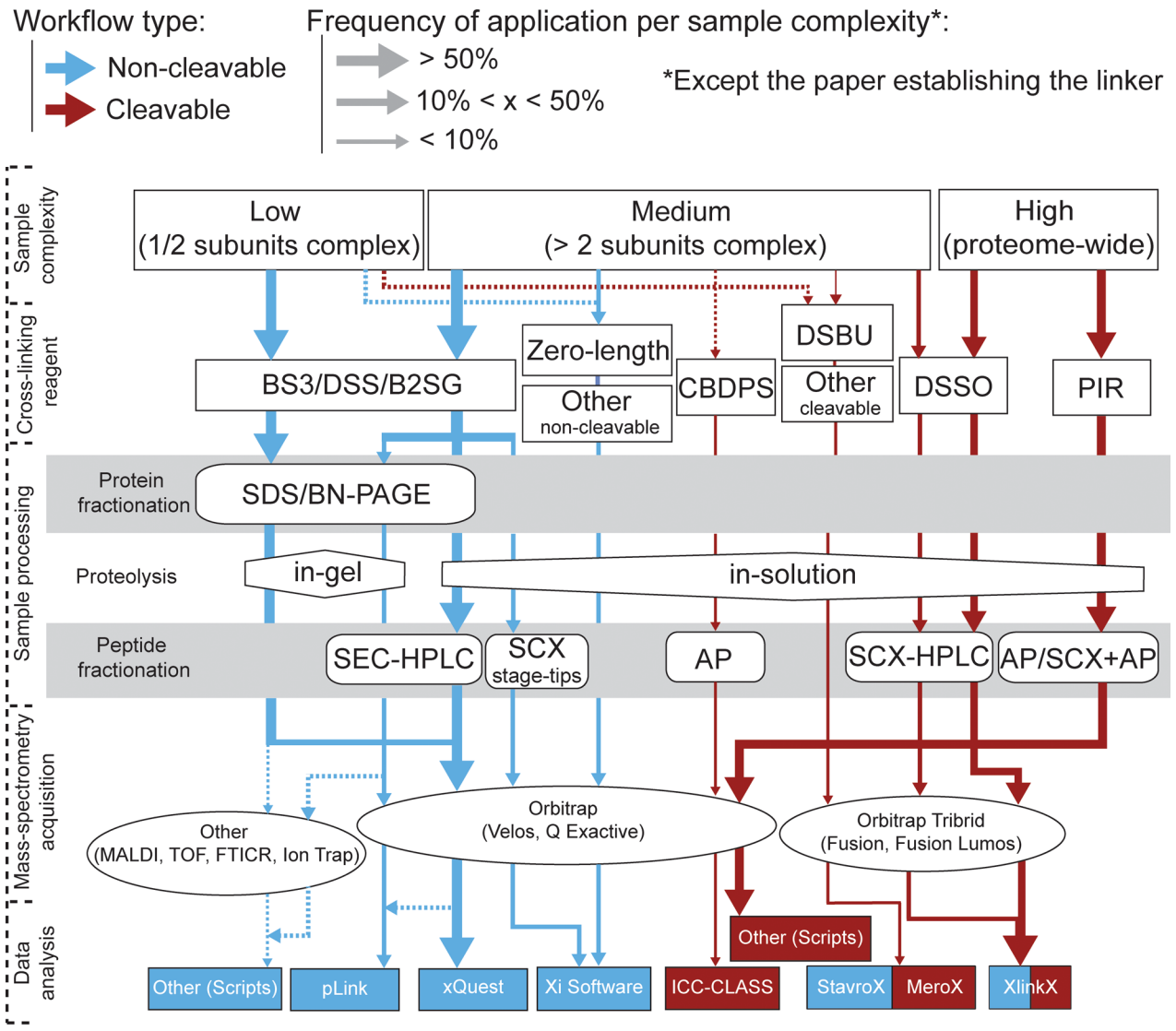

Figure 6. Flowchart quantitatively describing the main workflows used in XL-MS over the last 10 years. Comparison between experimental workflows exploiting mainly noncleavable (blue) and cleavable (red) cross-linking reagents. The thickness of the connecting arrows provides quantitative information, which is normalized to the studied sample type. Hybrid approaches are indicated by dashed arrows.

although it appears there are six major players receiving over time equal treatment. Of note here is that nearly one third of the software solutions were classified here as "other", which hides a myriad of options ranging from scripting to the use of search engines originally designed for normal peptide workflows like MaxQuant/Andromeda. The relatively recent emergence of cleavable cross-linking reagents is for example at play here, as developing laboratories are implementing data analysis strategies utilizing the extra information. This has meant that the existing tools could not be used out-of-the-box, but required, e.g., scripts to tie the results together in a final set of cross-linked peptide pairs. At this point, it is difficult to predict where the field is heading or that, not unlike as has happened in shotgun proteomics, a myriad of options will remain in use since addressing different biological problems requires diversification of approaches. The number of applications of cleavable cross-linking reagents has not received more traction so far, which can be attributed to the fact that the traditional workflows using noncleavable linkers have become very popular among structural biologists. As expected, they are applied to complex protein environments, but these types of studies have not been widely applied so far. Whether this will occur more frequently in the near future is difficult to predict, although we think this is an interesting approach to uncover protein−protein interactions on a global scale and more importantly prove their existence in a close-to-native state without the need of extensive purification procedures. Overall, the picture that emerges for choosing an optimal strategy is quite complicated. To ease the process of selecting a strategy, we have attempted to summarize everything in a flow chart (Figure 6). This flow chart summarizes Table S1 and provides quantitative information highlighting the most common workflows depending on the sample complexity, which can be used to retrieve specific case studies applied to biological systems similar to the one of interest. Note that publications establishing the linker were not considered while preparing this figure as initially all included reagents were tested on low-complexity samples, thus producing potential biases in the interpretation.

There are also new developments in the field that have so far not shown up in this analysis as they are simply too recent. We place the weight here on the development of novel cross-

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linking reagents, for which we discuss below azide-A-DSBSO,[63] Leiker,[12] and PhoX,[13] all reagents that incorporate an enrichment handle to select the cross-linked peptides directly out of the complex background of normal peptides. Such reagents have the promise to greatly improve the analytical depth at reduced LC−MS measurement times by abolishing extensive pre-fractionation steps. This ignores developments like the incorporation of new amino acid reactive groups on the scaffolds of existing cross-linking reagents as well as the photoactivatable reagents. For now, we believe such reagents will have a limited use based on the observation that the vast majority of the applied cross-linking reagents target lysine residues.

(I) Azide-A-DSBSO is a gas-phase cleavable cross-linking reagent based on the DSSO line of reagents (Figure 7a). Through an acetal ether bond that can be cleaved using acids, the spacer is connected to an azide group to further functionalize the cross-linked peptides with a biotin handle through click chemistry. This has the advantage that the reagent retains a relatively small footprint during the cross-linking reaction, which can be a consideration when very tight interaction interfaces need to be uncovered. Cross-linked peptides are then after the click reaction and avidin enrichment released by acidic cleavage, resulting in a three-step enrichment procedure. (II) Leiker is a noncleavable isotope-coded reagent with a biotin handle already attached through an azobenzene moiety (Figure 7b). After the enrichment of cross-linked peptides using avidin, cross-linked peptides can be released through chemical cleavage of the azobenzene, resulting in a two-step enrichment procedure. Although noncleavable, this linker has been applied successfully to highly complex lysates. (III) Difficulties of working with biotin and sample loss during the click-step have forced a new avenue to be explored. The third reagent, PhoX, incorporates a phosphonic acid on a noncleavable cross-linking reagent to provide an alternative to biotin (Figure 7c). The phosphonic acid moiety is a stable mimic of a phosphate group. Therefore, PhoX cross-linked peptides can be enriched using well-established phosphopeptide enrichment strategies such as Fe-IMAC, which are already implemented in most proteomics laboratories. Its small footprint and easy elution from IMAC preclude the need for incorporation of a chemical cleavage site, resulting in a one-step enrichment procedure. Competing molecules for IMAC enrichment such as phosphopeptides and nucleic acids can selectively be removed by phosphatase or benzonase treatment, as PhoX remains stable under these conditions. Also, PhoX has already been successfully applied to complex lysates.[13]

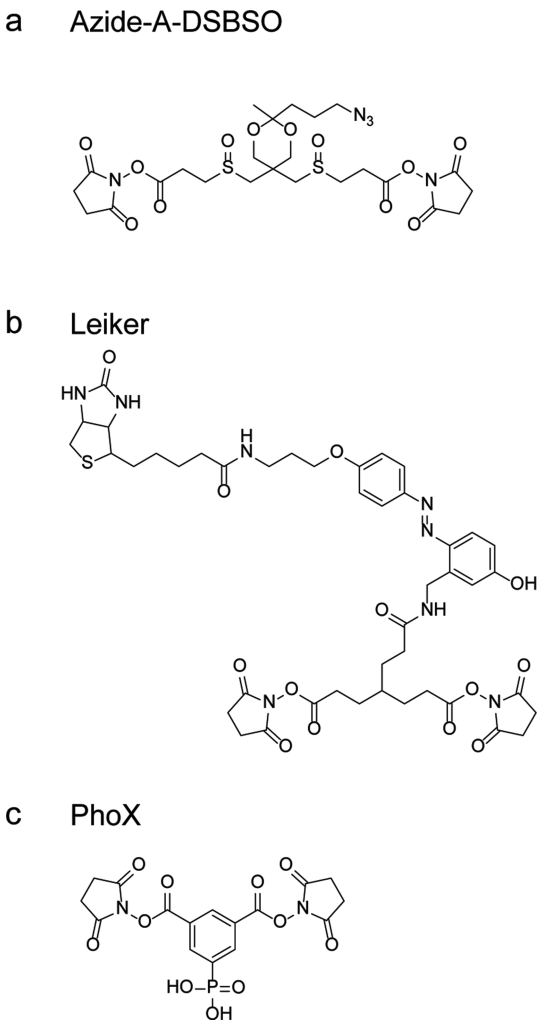

Figure 7. Novel approaches. (a) Azide-A-DSBSO, a functionizable DSSO variant that can be extended with a biotin enrichment handle. (b) Leiker, an isotope-coded noncleavable cross-linking reagent incorporating a biotin enrichment handle. (c) PhoX, a noncleavable cross-linking reagent incorporating a phosphonic acid amenable to IMAC enrichment.

Overall, XL-MS is a fast-developing field with a stable core of workflows that are preferentially applied. This does not preclude new developments from taking the spotlight in this exciting field, and we are looking forward to the coming years with hopefully a number of the new developments gaining traction to tackle biological questions of increasing complexity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jasms.9b00085.

Description of Table S1 and references (PDF)

Table S1 containing all of the references collected for this review (XLSX)

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Author Contributions

R.A.S. and A.J.R.H. conceived of the work. B.S., P.A., and R.A.S. performed the literature search and analysis of the publications. Chemistry by B.S., biochemistry by P.A., and statistics by R.A.S. All authors critically read and edited the manuscript.

Author Contributions

§B.S. and P.A. contributed equally.

Notes

The authors declare no competing financial interest.

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