Evolution of Chemistry for Precision Engineering of Proteins

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https://doi.org/10.51167/acm00047

A chemical toolbox for protein bioconjugation drives the innovation at the biology-medicine interface. It provides an alternative to the biochemical approaches while enabling access to a much more significant proteome segment. However, "precision" was often elusive from the bioconjugation methods till recently. The increasing knowledge of core principles for bond formation and dissociation with proteins has led to multiple breakthroughs in recent years. It also unraveled protein-driven reactivity and selectivity attributes unknown to small molecules' chemical reaction repositories. This research field promises to address the growing demands in protein-based diagnostics and therapeutics.

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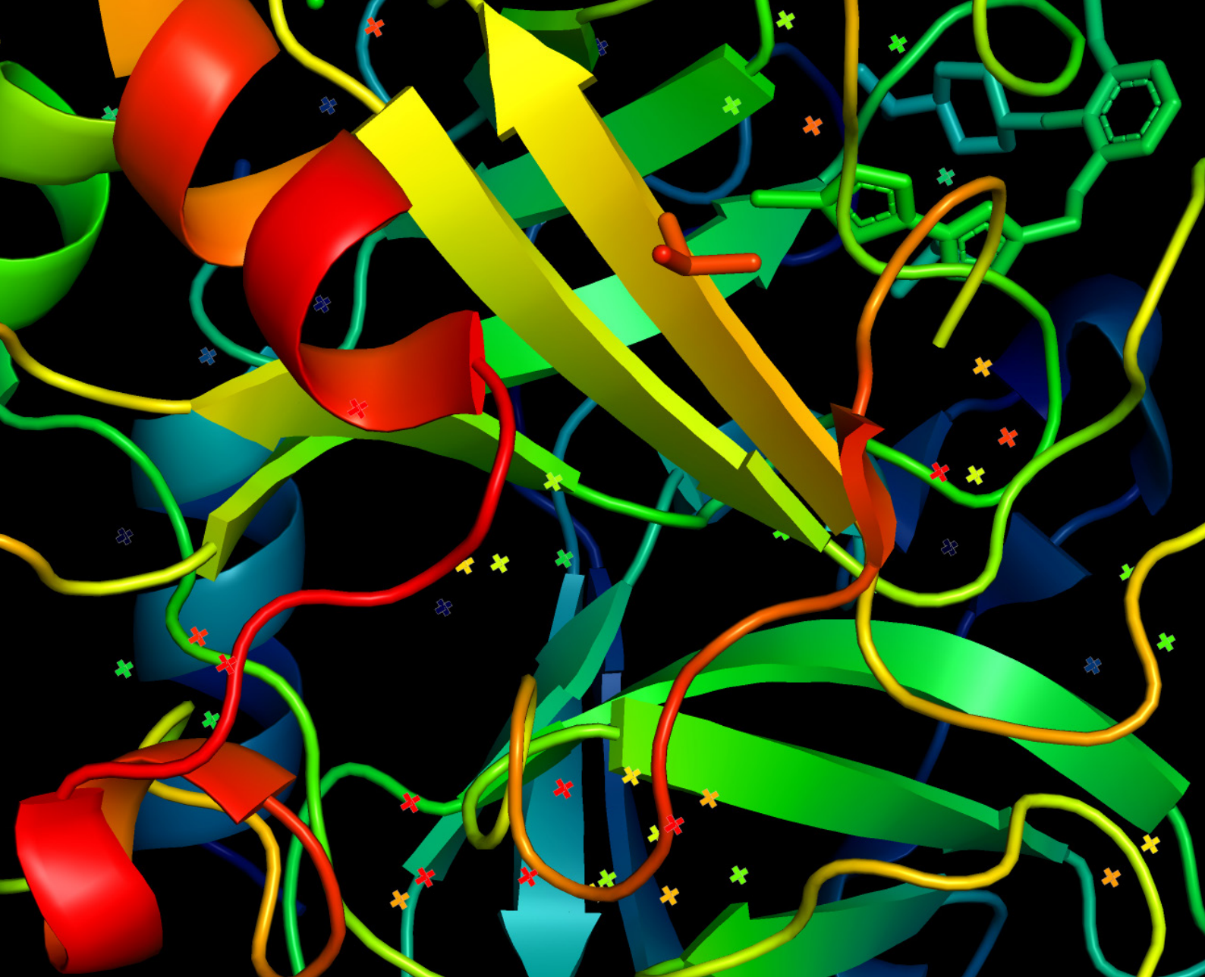

Introduction

Proteins are one of the most versatile complex biomolecules in living organisms as they play critical roles in biological activities. Understanding their function in the cellular milieu allows us to investigate biological pathways comprehensively. Retrospectively, proteins are often the first choice for phenotypic connections when the cellular machinery behaves abnormally. Hence, they also become the target of choice for therapeutic interventions. The protein comprises a pool of nucleophilic amino acid residues from a chemical perspective. The understanding and deconvolution of the broad spectrum of nucleophilicity offered by these functionalities are critical for their selective modification. This article overviews chemical technologies and the emerging principles from this perspective. The field impacts biophysical and biochemical research, diagnostics, protein-based therapeutics, and small molecule covalent inhibitor development for precision therapeutics (Fig. 1).

Biophysical investigations require the covalent tagging of proteins to study their structure, dynamics, and biomolecular interactions.[1] A promising approach for the same involves tethering protein with the probe of interest.[2,3,4] For example, the installation of fluorophore and fluorescence polarization drives the investigation of protein-protein, protein-peptide, and protein-oligonucleotide interactions.[5] Besides, Förster resonance energy transfer (FRET) allows the investigation of protein folding and biochemical reactions.[6] The bioconjugation of fluorescence reporters also enables the ***biochemical investigations*** of dynamic chemical messengers. As a result, they facilitate activity-based sensing of ROS[7] and binding-based sensing of calcium and other metal ions.[8]

From the translational standpoint, early diagnosis of the disease substantially impacts the patient's prognosis. Besides,

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point-of-care diagnostics gain attention for improving the reach or tackling the spread of infectious diseases. Protein covalent modification strategies render rapid detection biosensors.[9] Bioconjugation of antibodies or proteins with specific enzymes leads to the development of enzyme-linked immunosorbent assay (ELISA), which provides a valuable tool for diagnosing viral infection.[10] Immobilization of antibodies or proteins on solid surfaces results in kits for lateral flow immunoassays and used to develop portable diagnostics devices.[11] The COVID rapid antigen test kits, glucose meter, and pregnancy kits are a few selected examples.[12] Antibody-fluorophore conjugates (AFCs) provide tools for image-guided surgery to help surgical oncologists to visualize and differentiate the tumor cells.[13]

Bioconjugate therapeutics involves proteins covalently attached to a drug or a property regulator. For example, the PEGylation of therapeutic proteins regulates their solubility, circulation half-life, proteolytic resistance, bioavailability, and cytotoxicity for effective control over pharmacokinetics (PK).[14] Over the last two decades, the knowledge of cancer immunotherapy biomarkers has also accelerated the use of antibody-drug conjugates (ADCs) for directed cancer chemotherapeutics. It comprises toxins tethered with a monoclonal antibody which selectively targets the overexpressed antigens in cancer cells and improves the therapeutic index in clinical practice.[15] Another rapidly growing segment comprises conjugate vaccines typically constituted of a carrier protein or virus-like particle (VLP) conjugated with the antigen. For example, the conjugation of a carrier protein to the carbohydrate-based antigen in a glycoconjugate vaccine could significantly impact the immune response duration. Besides, the control over bioconjugation provides the window to regulate the antigen/carrier protein ratio and structure to impact the immune response effectively.[16] The conjugation of antigen to a VLP, virus-mimic without genome, also yields successful conjugate vaccine candidates.[17] The bioconjugation toolbox is expected to meet the daunting task of providing flexibility with homogeneity, conjugation site, stability, linkers, and payloads.

Additionally, the cell-drug conjugates also ensure targeted delivery with prolonged circulation and reduced toxicities.[18] For example, the conjugates with immune, blood, and stem cells have progressed toward antitumor therapy.[19] Another exciting development revolves around the potential use of the electrophilic pool from bioconjugation in developing covalent inhibitors in drug discovery.[20] Additionally, coupling this concept to protein degradation platforms, e.g. PROTACs, will further extend the druggable proteome beyond enzymes.[21] While the approaches like chemoproteomics fuel it at this stage, the bridge between these segments is expected to grow stronger in the coming years.[22]

Challenges for chemistry

The modification of a residue in protein requires the understanding of functional group distribution and its attributes. The chemical transformation used to architect a bond is inspired by classical organic chemistry. However, it becomes challenging to translate most of them to proteins as it comes along with associated restrictions on the reaction conditions. Hence, optimum utilization of limited organic chemistry acumen under physiological conditions becomes essential (Fig. 2). At first, getting practically useful kinetics at low micromolar

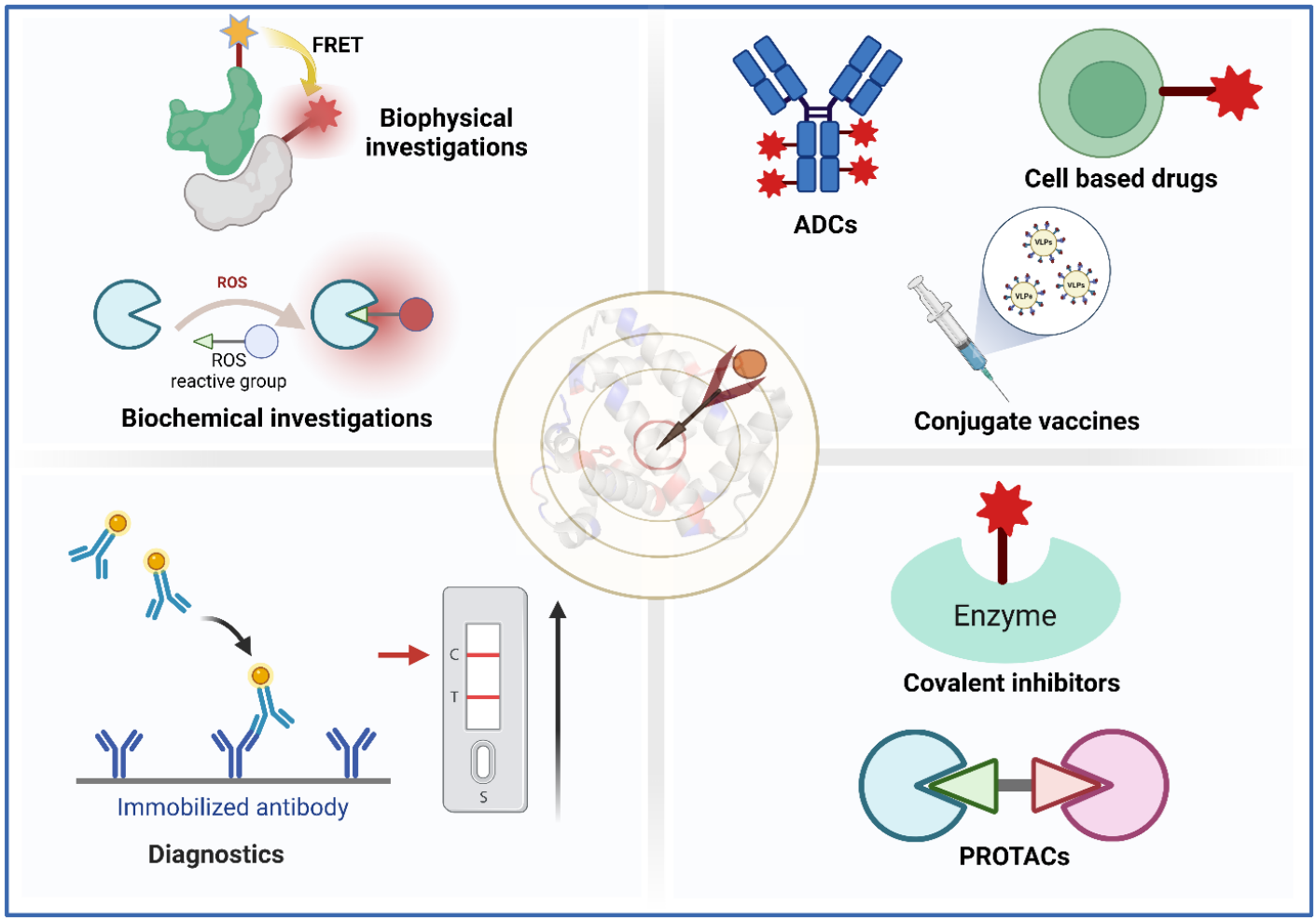

Figure 1. Translational opportunities for chemical technologies enabling precision engineering of proteins.

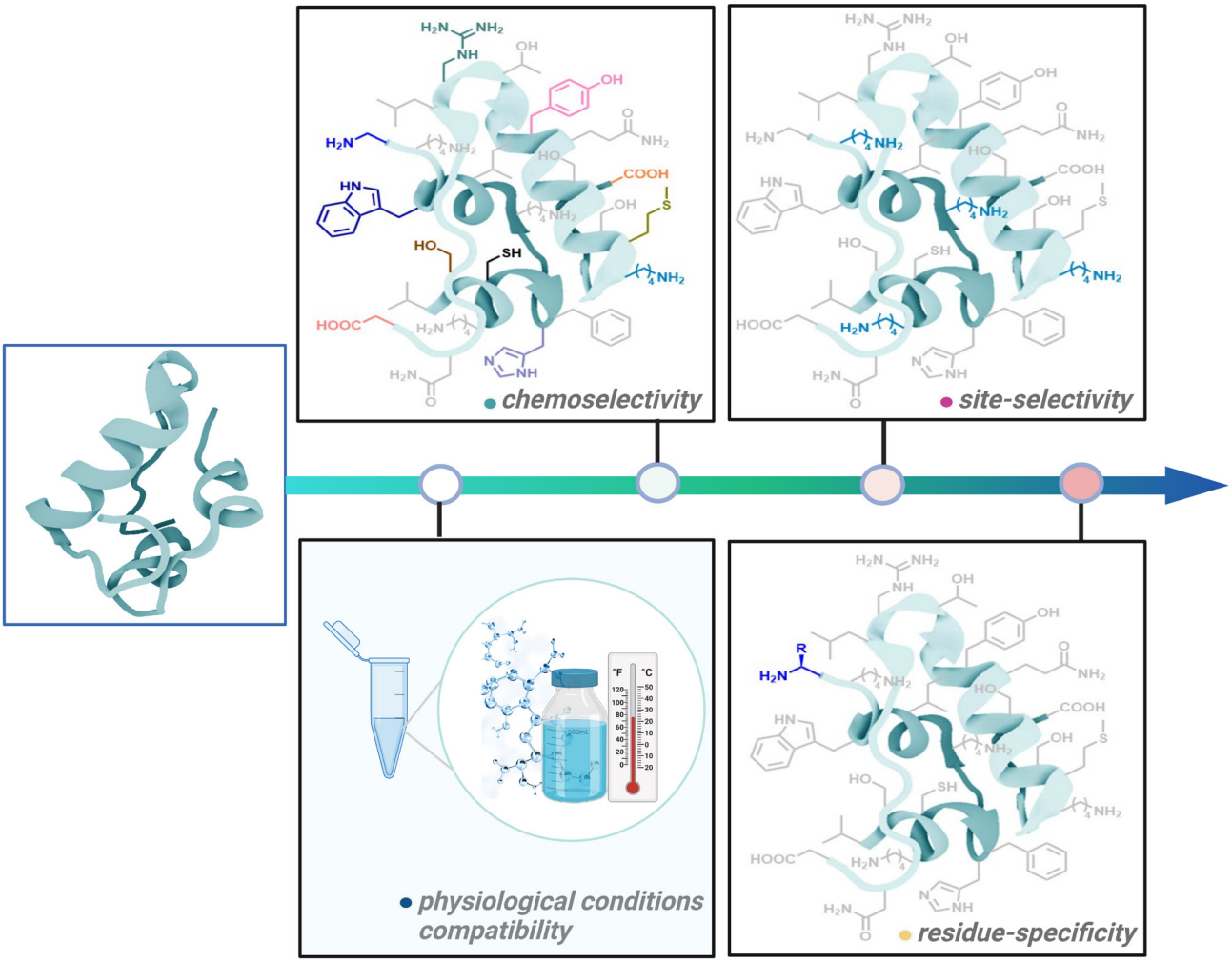

Figure 2. Roadblocks: redefined reactivity and selectivity landscape.

concentration is essential. The solubility of bioconjugation reagents and their stability in the presence of nucleophilic water as the solvent are necessary. The organic solvent compatibility of substrates for construction of conjugation reagents enables their synthetic manipulation and purification. However, it is also the reason why most of the reagents display low aqueous solubility. Flexible methods for late-stage installation of hydrophilic handles could address such cases. The assistance from organic solvents to solubilize the bioconjugation reagent is acceptable till its final concentration is not irreversibly denaturing the protein. Some electrophilic reagents also constitute reversible covalent bonds with multiple proteinogenic residues. Hence, the ideal stoichiometry of the bioconjugation reagent is an unpredictable parameter and necessitates case-to-case optimization. Additionally, the proteins with a source-dependent variation of post-translational modifications, such as antibodies, present an altered landscape of solvent accessibility for the residues. The change in the microenvironment impacts reactivity and selectivity and needs a careful assessment to maintain batch-to-batch consistency.

The selectivity in protein bioconjugation is a multifactorial problem (Fig. 2). The reactivity order is defined by the combination of pKa and solvent accessibility and not alone by the prior, making it difficult to predict with confidence. However, the kinetically driven bioconjugation can assist in identifying it along with the reactivity hotspot or the most preferred site. Additionally, the single-site modification in practically useful efficiency presents a combination of challenges. If an electrophilic reagent is added to a protein, it must differentiate one proteinogenic residue from the others. Such a chemoselective electrophile must subsequently target a residue in the presence of its multiple copies to achieve the site-selectivity. On the other hand, the residue or site-specificity demands such an electrophile to identify a unique combination of functionalities emerging from proteinogenic amino acids.

Post bioconjugation, separating modified protein from its native version could offer additional value. The subtle modifications do not alter the properties enough for their separation and require the installation of dedicated tags or handles. On the other hand, the bioconjugation reagent is typically a small molecule that can be separated conveniently through dialysis, chromatography, or centrifugal spin concentration through a membrane.

Mass spectrometry (MS) offers a powerful analytical tool for the rapid and unambiguous analysis of bioconjugation (Fig. 3). The MS of the mixture containing unreacted and modified protein(s) confirms the conversion. The accuracy of this estimation depends on the relative size and nature of protein and conjugated tags. The subsequent analysis requires proteolytic digestion of the bioconjugate. The MS-based mapping of this mixture helps identify peptide(s) with an additional bioconjugation reagent tag. The confidence in homogeneity depends on the extent of sequence coverage. The inefficient proteolysis and self-degradation of protease could negatively impact the latter. Also, the concentration disparity of peptides in the

digest, different ionization efficiency, and peak suppression plays a role. The MS-MS (MS2) of the tagged peptide in the next phase confirms the conjugation site.

The above analytical sequence needs a few additional steps for proteins with higher structural complexity. One of the common examples involves antibody-conjugates such as antibody-fluorophore conjugates (AFCs) or antibody-drug conjugates (ADCs). While the AFCs cater to image-guided surgery, ADCs have been addressing the demands of directed cancer chemotherapeutics. The monoclonal antibodies (mAbs) involved in these bioconjugates weigh over 150 kDa and comprise four subunits, i.e., two light (LC, ~25 kDa each) and two heavy chains (HC, ~50 kDa each). The mass spectrometer attribute in the given mass range presents the first challenge.

The post-translational modifications (PTMs) of mAbs contribute to sample heterogeneity. However, enzymes such as PNGase F[23] and Endo S[24] can reduce it by cleaving the glycan component. Another complication emerges from heterogenous bioconjugation, making it difficult to confidently establish the extent/site of labeling and batch-to-batch reproducibility. Besides, a reasonable mass difference (≥ 300 Da) between mAb and its conjugate is necessary to reduce the peak overlaps. The probe to antibody ratio calculation can be done at this point for analytes rendering well-resolved data. Further, the disulfide reduction and analysis of resultant subunits provide insight into the extent of modification in HC and LC, respectively. Also, it can confirm the tag-to-antibody ratio in the absence of intact antibody-conjugate MS resolution. The LC and HC separate on liquid chromatography conveniently. While LC analysis is straightforward, the PTMs in HC suppress its ionization. However, the enzymatic removal of glycans can help if needed. It is essential to note that the mono-labeled antibody can have more than one site involved in the conjugation leading to site-heterogeneity. Hence, peptide coverage is a crucial parameter in establishing homogeneity. The primary sequence coverage of Fab CDRs (Complementarity Determining Regions) and immune-cell recruitment Fc domain is critical to ensure that they are unperturbed. The proteases or combinations (e.g., trypsin with α-chymotrypsin) can generate the dataset for high primary sequence coverage. Maspecter[25] sensitivity boosters can also enhance the peptide coverage if required. Finally, the MS-MS confirms the exact site of drug conjugation. The optimal range for sample concentration, injection volume, liquid chromatography, and MS methods play an essential role in data reproducibility.

Chemoselective chemical methods

The landscape of chemoselectivity is predictable and better understood for molecules with limited functional groups. In a protein, the copies of each residue experience a different microenvironment. As a result, it presents a broad spectrum of reactivity for each type of residue. Hence, it eliminates the gaps in the reactivity profile and is a substantial factor that challenges electrophiles to display conserved selectivity. At the same time, each site's unique reactivity creates an opportunity to target them selectively. Understanding this redefined landscape of reactivity is essential to harnessing selectivity in protein bioconjugation. Besides, the nucleophilic residues such as lysine, arginine, or histidine, exist in protonated form under physiological conditions. While this could reduce their reactivity, it also ensures their high solvent accessibility. This section outlines the selected electrophiles that display chemoselectivity with most of the isolated proteins (Fig. 4).

Carboxylates are displayed by aspartic acid, glutamic acid, and C-terminus in a protein. The low nucleophilicity and high frequency make it a difficult target for bioconjugation. The diazo compounds (**1a**, Fig. 4) present an appropriate electrophile for O-alkylation to render esters under physiological conditions.[26] The reactivity of precursors and stability of the product requires careful pH control. The carboxylic acid activation is well established for amide synthesis. However, it faces multiple competitors in proteins. For example, EDC/N-hydroxysulfosuccinimide (**1b**) mediated conjugation establishes participation of multiple residues.[27] The photoactivated diaryltetrazole (**1c**) reacts with the carboxylic acids, albeit in low conversions.[28] On the other hand, 3-phenyl-2H-azirine (**1d**) enables chemoselective modification of carboxylate under mild conditions.[29]

Arginine is a challenging target for the electrophiles in the presence of amines and thiols. However, if an electrophile offers a reversible reaction with the more reactive competitors, the equilibrium can drive the reaction towards selective Arg modification. It encouraged the reaction of Arg with the dicarbonyl compounds like phenylglyoxal.[30] Later, PEGylated oxoaldehyde (**2a**, Fig. 4) was designed to target Arg in lysozyme.[31] The conjugates were stable

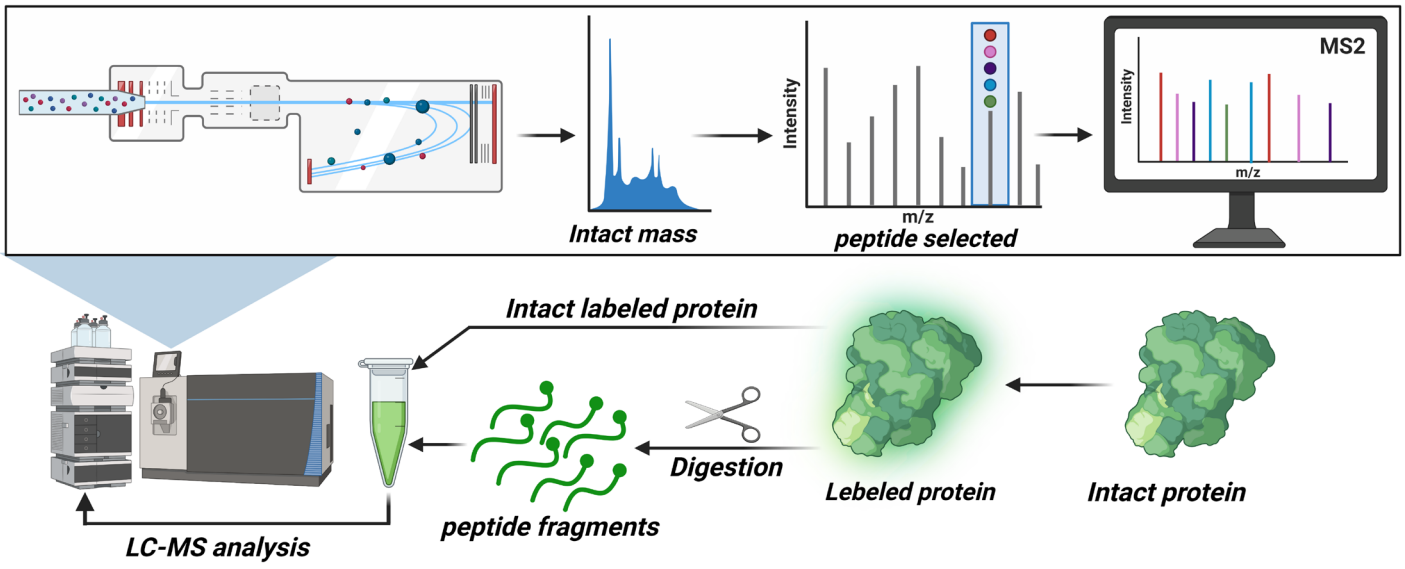

Figure 3. Roadblocks: analytical tools

toward hydroxylamine over a broad pH range. Recently, dibenzocyclooctendiones (**2b**) were established to be Arg-specific, enabled by a benzylic acid rearrangement reaction.[32] The reactions were performed in 90% organic solvent with peptide-based models, and it will be interesting to see the translation to proteins under physiological conditions.

Histidine possesses a heteroaromatic imidazole ring that exhibits moderate nucleophilicity, which falls behind that of Lys and Cys. The His modification often involves a polarized bond that is more appropriate for reversible covalent bioconjugate-based applications. In one of the initial results, 4-hydroxynon-2-enal could target His in the absence of a free Cys.[33] Later, epoxides (**3a**, Fig. 4) evolved as one of the most promising handles for His targeting under mild alkaline conditions.[34] This selectivity also translates to an epoxide coupled with an alkene (**3b**), where the conjugation initiates with Michael addition and ends with the epoxide ring opening.[35] Going beyond the N-centered bioconjugation has proven challenging. The C4-alkyl-1,4-dihydropyridine reagents (**3c**) could promote a radical-mediated C-H alkylation with His under visible light.[36] However, the successful translation to proteins under practically applicable conditions is yet to be realized.

Tryptophan also becomes an interesting target due to its low occurrence in the proteome. The indole in the side chain makes it a convenient target. For example, vinyl metallo-carbenoids (**4a**, Fig. 4) using rhodium catalysis offer Trp-selectivity with peptides over a broad pH range (2-7).[37] However, the yields are moderate in general. Also, the poor solvent accessibility of Trp in most cases necessitates protein denaturation. The in-situ generation of trifluoromethyl radical (NaTFMS, catalytic TBHP, **4b**) results in Trp-labeling at the C2 position.[38] The selectivity challenges can be addressed to some extent by lowering the reaction temperature. Further, Au (I)-catalyst and Waser reagent (TIPS-EBX, **4c**) render regio- and chemoselective Trp C2-ethynylation of protein.[39] It requires the use of organic solvent along with water to achieve efficiency. Besides, the electron-responsive N-carbamoylpyridinium (**4d**) salts can result in Trp modification in the presence of UV and glutathione under

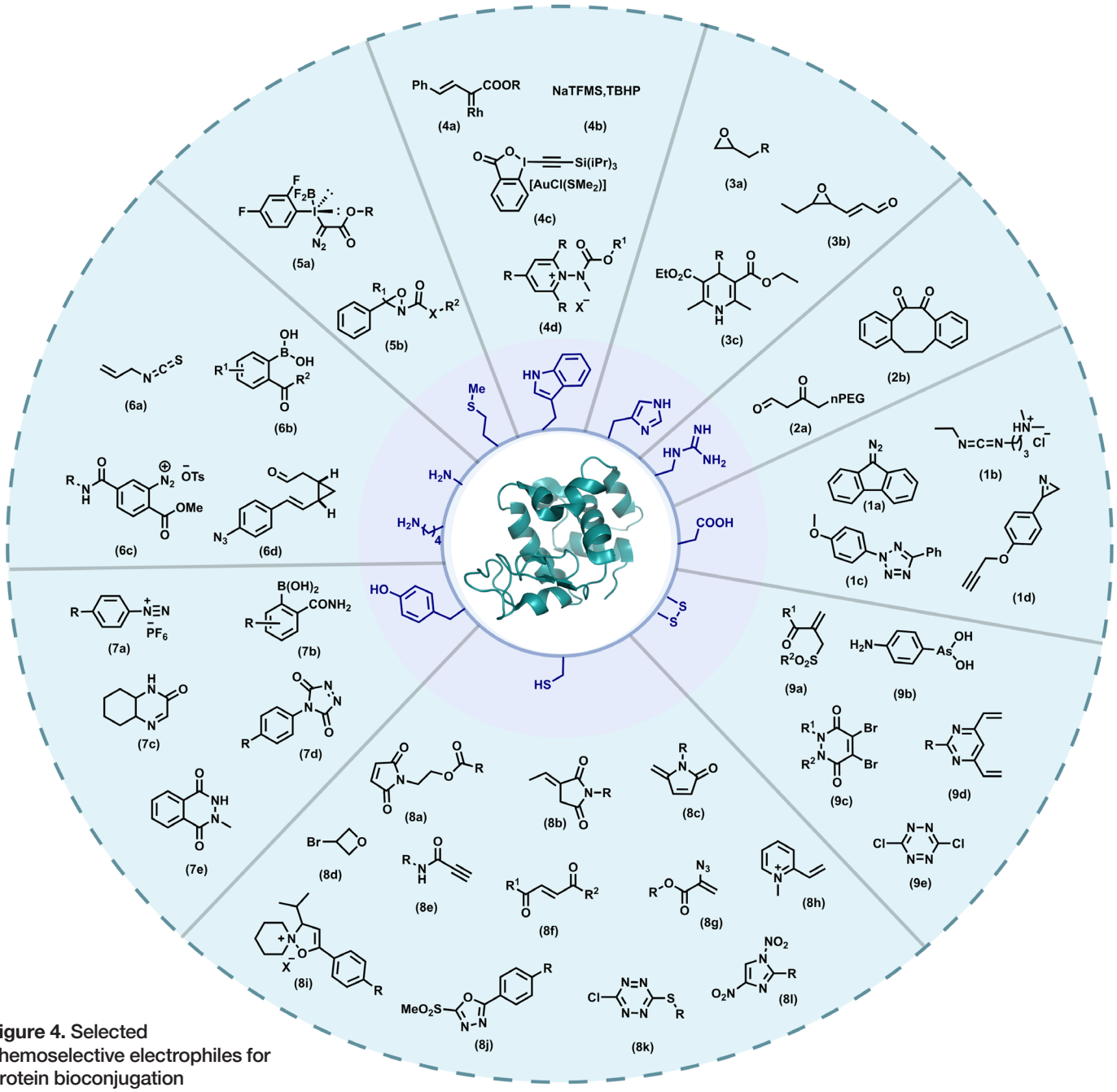

Figure 4. Selected chemoselective electrophiles for protein bioconjugation

aqueous conditions.[40] Importantly, the reaction conditions are well-tolerated by other redox-active amino acids.

Methionine's sensitivity to the redox environment and lack of solvent accessibility makes it a challenging target. Hence, the choice of protein and reagent becomes critical. The hypervalent iodine reagent (**5a**, Fig. 4) alkylates methionine by forming sulfonium conjugate.[41] It can render good kinetics with low relative stoichiometry, but side reactions are tough to avoid. On the other hand, the redox-activated chemical tagging (**5b**) of protein using oxaziridine-based reagents offers good selectivity.[42] It initiates with a nucleophilic attack on the N or O of oxaziridine. The subsequent intramolecular rearrangement results in the release of sulfimide/aldehyde or sulfoxide/imine, respectively. While the former is desired, the latter can contribute to the side product.

Lysine and N-terminus amine: The high frequency, solvent accessibility, and reactivity make primary amines a favourite target for chemoselective reaction. Acylation using activated esters is commonly employed.[43] While Cys could supersede its reactivity, the difference in stability of thioester versus amide helps. In a conceptually similar manner, the reversible thiol adduct with isothiocyanate also creates an opportunity for irreversible Lys modification. For example, the reaction of allyl isothiocyanate (**6a**, Fig. 4) with bovine serum albumin renders a chemoselectively constructed end-product.[44] The reversible reaction of aldehydes with competing residues while constructing an electrophilic intermediate with amine also paves the way for their chemoselective modification. The Ir-catalyzed reductive alkylation[45] and iminoboronate formation (**6b**)[46] present a few examples. Coupling with diazonium terephthalates (**6c**) where an initial reaction with diazonium ion is followed by intramolecular capture by ortho-ester moiety, provides another interesting approach.[47] In another case, divinylcyclopropane-cycloheptadiene (**6d**) uses intramolecular rearrangement for irreversible amine modification.[48]

Tyrosine: The low frequency and solvent accessibility of tyrosine make it a reasonable target when available in a protein. The rich electrophilic aromatic substitution reactions with a phenolic group create opportunities. For example, the diazonium coupling reaction (**7a**, Fig. 4) is used to install chemically orthogonal handles for subsequent installation of probes.[49] Another strategy involves three-component complexation of Rh (III), boronate (**7b**), and a tyrosine residue.[50] The metastability of the inorganic linkage and its sensitivity towards the nucleophilic redox mediators demands further attention. The water-stable cyclic imines (**7c**) could offer Tyr-selectivity over a wide pH range, albeit with low reactivity.[51] Interestingly, the ene-type reactions involving 4-phenyl-3H-1,2,4-triazoline-3,5(4H)-diones (PTADs) deliver excellent reactivity while retaining the chemoselectivity.[52] The electrophilic isocyanate generated in this reaction could impact the selectivity. On the other hand, diazodicarboxyamide (**7d**) can achieve it without generating an electrophilic species.[53] Further, hemin-activated luminol (**7e**) also renders Tyr-selectivity under H_2O_2-assisted oxidative conditions.[54]

Cysteine is one of the most exploited targets due to its high nucleophilicity and low frequency. Its redox activity, capability to construct reversible covalent bonds, and presence in the catalytic domain of enzymes make them valuable contributors to diverse functions. The pKa and soft nucleophilic thiol render an uncompromised reactivity with electrophiles with diffused electron density under physiological conditions. The α-halocarbonyl compounds, such as iodoacetate[55] or iodoacetamide,[56] are commonly used for Cys-alkylation. The polarized double bonds also present excellent electrophilic warheads for this purpose. For example, maleimides serve as a versatile handle for chemoselective modification of Cys (**8a**, Fig. 4).[57,58,59] However, these bioconjugates are pH-sensitive, and retro-Michael reaction, external nucleophile, or intramolecular aminolysis often triggers the C-S bond dissociation.[60] Exocyclic maleimides (**8b**) address the problem to a reasonable extent.[61] The substrates promoting ring hydrolysis post-Michael addition also offer a viable solution.[62] The C-S reversibility can be used for specific applications if chemically orthogonal conditions promote bond formation and dissociation. For example, 5-methylene pyrrolones (**8c**) render rapid C-S bond formation under neutral pH dissociating through thiol exchange (pH 7.5) or retro-Michael reaction (pH 9.5).[63,64] Vinyl sulfones and sulfoniums offer relatively more stable conjugates, but chemoselectivity challenges often accompany them.[65,66] Oxetane (**8d**) offers another category of reagents suitable for Cys-alkylation.[67] The terminal alkenes[68] and alkynes provide an excellent handle for thiol-ene (TEC) and thiol-yne[69] coupling reactions. The light-induced radical formation followed by anti-Markovnikov addition to the alkene results in a thioether that another round of photoirradiation can reverse.[70] Thiol-yne coupling follows the radical mechanism like TEC but involves adding two thiol groups to the alkyne. The polarized alkynes, including alkynoic amides (**8e**), esters, alkynones, and 3-arylpropionitriles, also render Cys modification.[71,72] The recent utilization of carbonylacrylic reagents (**8f**),[73] azidoacrylates (**8g**),[74] quaternized vinyl/alkynyl-pyridine reagents (**8h**),[75] isoxazolinium reagents (8i),[76] adds another set of promising candidates for thiol-targeting. Further, methylsulfonyl benzothiazole (MSBT)[77] inspired the use of phenyltetrazole (**8j**) and phenyloxadiazole.[78] They promote Julia-Lythgoe olefination through a Smiles rearrangement on the heteroaromatic ring. Besides, a dichlorotetrazine derivative (**8k**) delivers Cys-selective nucleophilic aromatic substitution.[79] The 1,4-dinitroimidazoles (**8l**) react with Cys via cine-substitution under mild acidic aqueous conditions.[80] The thiol-disulfide exchange reactions use S-S reversibility and could promote targeting either of these groups.[81]

Disulfide becomes another viable target in the absence of free Cys residues. The α,β-unsaturated bis-alkylating reagent (**9a**, Fig. 4) rendered disulfide modification in human Interferon α-2b (IFN).[82] It involves the addition of a thiol to the conjugated double bond, Michael acceptor regeneration via elimination of sulphinic acid, and reaction with the second thiol. The product includes three carbon bridge between the two sulfur atoms. The entropy-driven affinity of trivalent arsenicals (**9b**) for dithiols led to another interesting route involving the sequential reduction-conjugation pathway.[83] Another interesting strategy involves the insertion of pyridazinediones (**9c**) into native disulfide bonds.[84] The divinyl-functionalised (**9d**) hetero-aryl linkers and dichlorotetrazine (**9e**) also proved their capabilities for disulfide re-bridging. [85, 86]

Site-selective chemical methods

The chemoselective methods often create an opportunity to differentiate a residue from its multiple copies to deliver site-selectivity. Besides, the localization regulators can bypass the requirement of chemoselectivity and deliver single-site bioconjugation even with a promiscuous electrophile. The magnitude of this challenge increases with the frequency and solvent accessibility of the residue. It also elevates with the decrease in the reactivity of the nucleophilic side chains.

Reactivity hotspots

Carboxylic acids are one of the least reactive residues. Additionally, their high abundance makes it challenging to target a single carboxylate. The photoredox decarboxylative alkylation approach used the difference in the oxidation potentials between Asp/Glu versus C-terminal carboxylate.[87] The employment of α,β-unsaturated carbonyl (**10a** , Fig. 5) ensured reversibility with Cys to avoid competition allowing selective trapping with the relatively high-energy C-terminal radical.

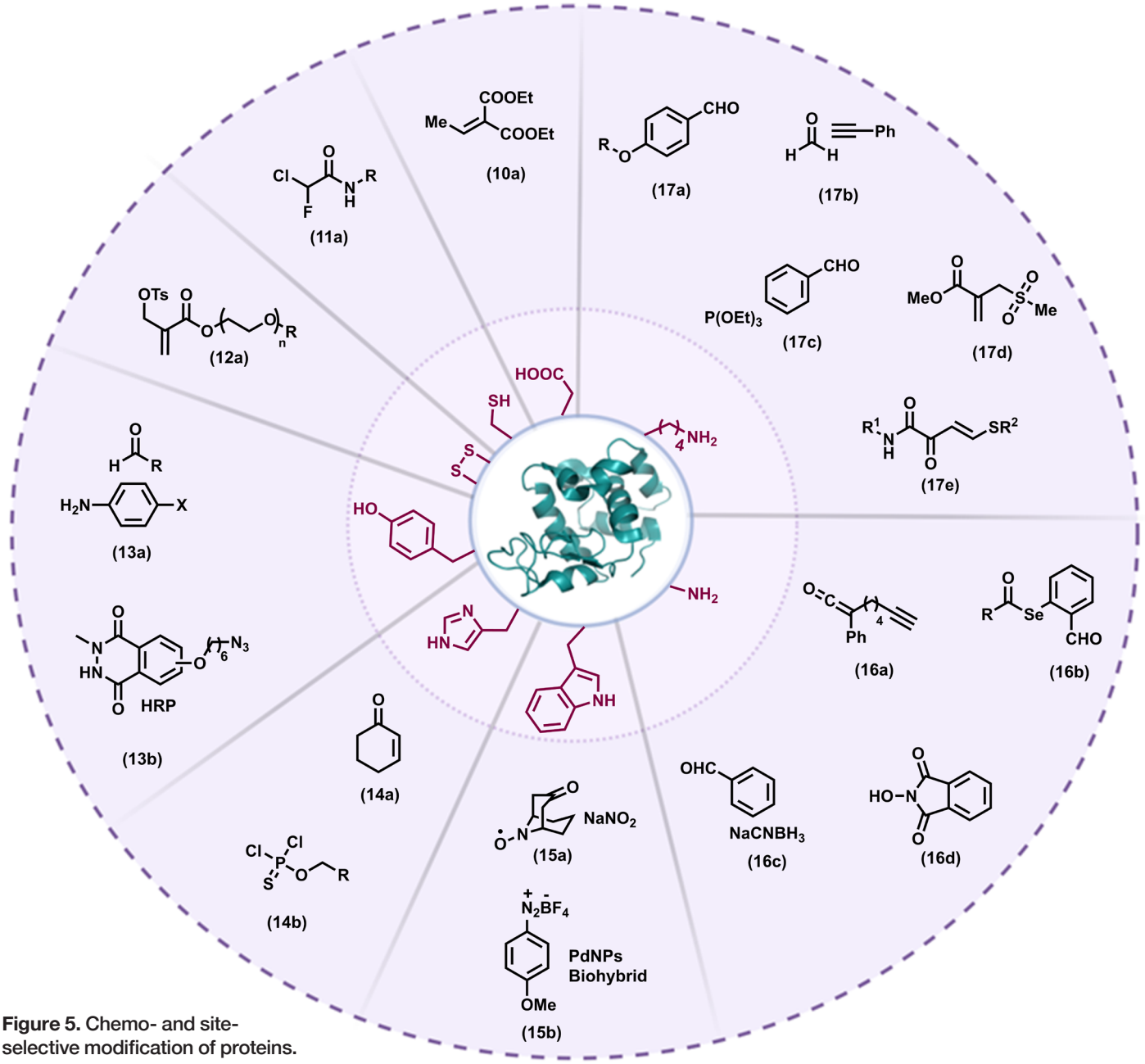

Figure 5. Chemo- and site-selective modification of proteins.

Cysteine: The low number of free Cys in proteome often provides it the bypass to site-selectivity challenge. Hence, the number of examples for site-selective Cys-modification is limited. The display of multiple free Cys would often require a mixture of proteins. Hence, differentiating one of them from the pool can render protein selectivity. These attributes connect this bioconjugation segment to the efforts to develop covalent inhibitors. In a representative case, α-chlorofluoroacetamide (**11a**, Fig. 5) renders selective modification of a Cys (non-catalytic) residue in kinase.[88] In another significant development, a ligand-bound electrophile irreversibly targets Cys in a common oncogenic mutant, K-Ras(G12C).[89]

Disulfide: Reducing the re-bridging disulfide bond creates a window for synthetic fragment insertion. Allyl sulfones (**12a**, Fig. 5) established single-site modification of solvent-exposed disulfide in lysozyme C.[90] After reducing the disulfide bridge by tris(2-carboxyethyl) phosphine (TCEP), the re-bridging by allyl sulfones involve a Michael addition, sulphinic acid elimination, and another Michael addition. The strategy translates well to deliver Fab and antibody conjugates.[91]

Tyrosine: The poor solvent accessibility and moderate frequency limit the number of proteins qualifying for Tyr conjugation. However, the same reasons make it a good candidate for single-site modification when available. The in-situ generated imines from aldehyde and electron-rich anilines provide an excellent intermediate to capture Tyr through a Mannich-type reaction (13a, Fig. 5).[92] The π-allylpalladium complex offer site-selective alkylation of surface-exposed tyrosine.[93] Further, a resin-tethered, highly reactive diazonium group enables catch-and-release for selective Tyr modification.[94] In another approach, single-electron transfer using horseradish peroxidase and electrochemical activation enabled Tyr-modification in the presence of an N-methylated luminol derivative (**13b**).[95]

Histidine is moderately abundant in the proteome, displays good solvent accessibility, and presents a difficult challenge for site-selectivity. TThe stability of imidazole-based bond in bioconjugate adds to the difficulties. stability of imidazole-based bond in bioconjugate adds to the difficulties. We demonstrated that a simple electrophile like 2-cyclohexenone (**14a**, Fig. 5) could render single-site His modification.[96] Here,

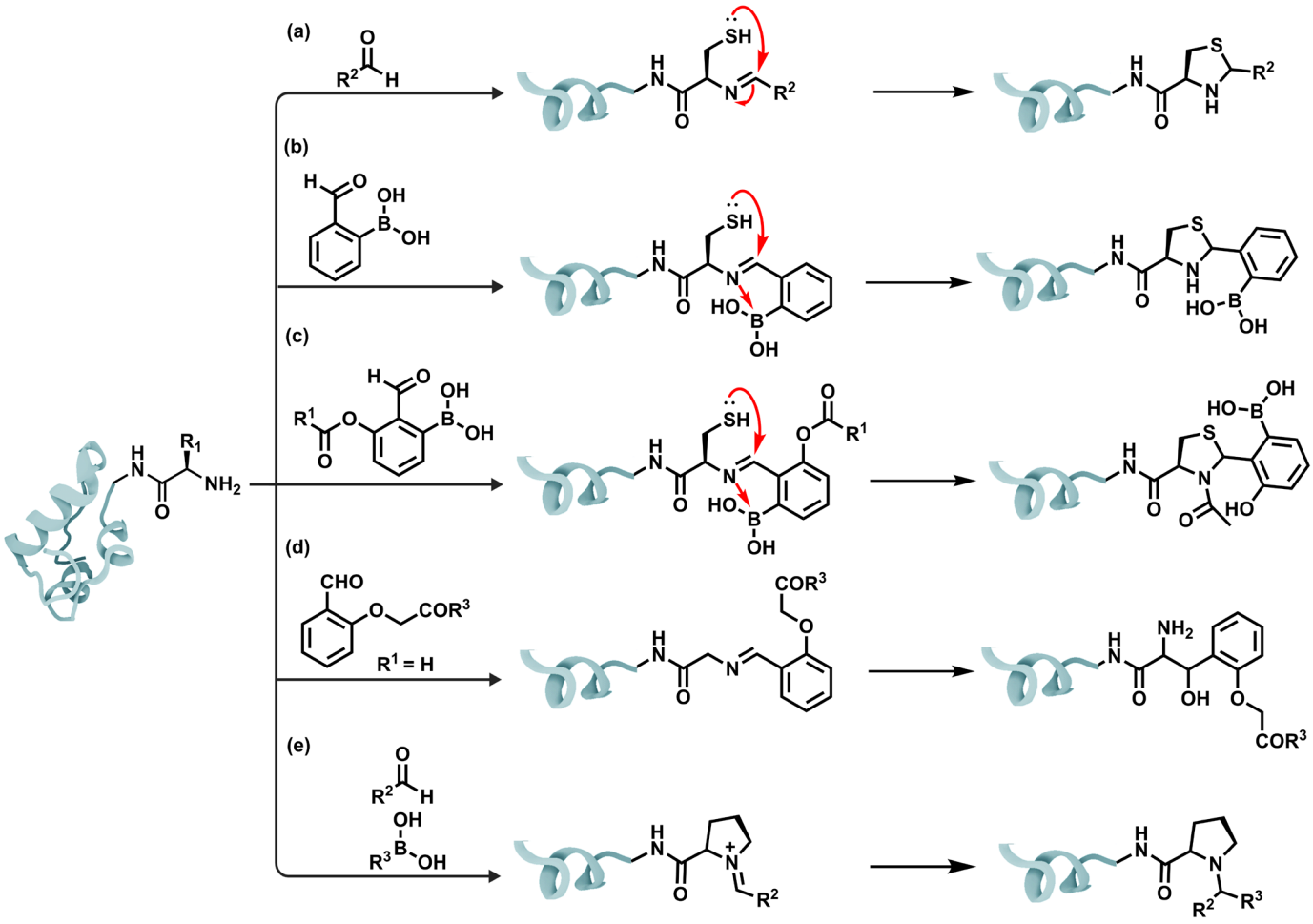

Figure 6. Residue-specific modification.

the hydrazone-oxime chemistry enables the late-stage installation of probes. Another critical development involved the thiophosphorodichloridate reagent (**14b**), which uses click chemistry for the late-stage installation of probes in the subsequent step.[97]

Tryptophan: The low frequency and moderate nucleophilicity left the site-selective modification of tryptophan as an underexplored segment. A recent effort involved the use of 9-azabicyclo[3.3.1]nonane-3-one-N-oxyl (keto-ABNO) (**15a**, Fig. 5) adduct in the presence of $NaNO_2$ and acetic acid.[98] The acid-promoted disproportionation and oxidation generate an oxoammonium intermediate. The conjugation is possible via a nucleophilic attack of the indole to oxoammonium intermediate. Another attempt involved a heterogeneous PdNP biohybrid catalyst (**6b**) that could enable the site-selective C-H arylation of Trp under mild aqueous conditions.[99]

Amines display high abundance coupled with high nucleophilicity to challenge site-selectivity. Their relative pKa and pH-controlled protonation come to the rescue while distinguishing N-terminus N^{α}-NH_2 and N^{ε}-NH_2 from all the lysine residues. The prior displays higher reactivity under physiological conditions, and the reactivity of the latter dominates at higher pH. The slow delivery of highly reactive NHS ester could deliver single-site N^{α}-NH_2 modification.[100] The diazo transfer reagents,[101] ketenes (**16a**, Fig. 5),[102] pyridoxal-5-phosphate[103] N-methylpyridinium-4-carboxaldehyde,[104] and selenobenzaldehyde ester derivatives (**16b**)[105] further establish the N-terminus selectivity. The **16b** involves capturing the amine by an aldehyde group followed by an acyl shift. The 2-pyridinecarboxaldehyde also initiates with the imine formation, where the subsequent intramolecular cyclization renders N-terminus imidazolidinone formation.[106] Besides, the intermolecular trapping of N-terminus imine with sodium cyanoborohydride (**16c**) can also be regulated to deliver site-selectivity.[107] In another example, triazole carbaldehydes form the N-terminus 4-imidazolidinone ring.[108] A Dimroth rearrangement can replace the triazole-4-carbaldehyde with an amine-functionalized labelling group.

We demonstrated that a two-centered electrophile (N-hydroxypthalimide, **16d**) renders single-site N-terminus modification.[109] An amphoteric intermediate capable of rendering a rate-determining irreversible intramolecular step serves as the backbone of this method. Further, we established that the N-terminus localization of an electrophile could supersede their inherent reactivity preferences.[110] It enabled single-site labelling of Nα-NH2 by sulfonate esters and epoxides, that inherently prefer carboxylic acids and histidine respectively. The kinetic preference for N^{α}-NH_2 also made it challenging to achieve Lys N^{ε}-NH_2 modification selectively. We established that the presence of a proximal nucleophilic backbone amide could distinguish the prior from the latter. The treatment of protein with an aldehyde (**17a**, Fig. 5) generates imine with all the solvent-accessible primary amines. Next, the N-terminus imine reacts further with the penultimate amide bond, resulting in imidazolidinone. Even though it is a reversible reaction, it is sufficient to block N-terminus amine as nucleophile or imine as an electrophile. It enabled the modification of a single Lys through formylating reagent generated by in-situ aldehyde auto-oxidation.[111] Also, it allowed a mixture of protein, aldehyde, acetylene, and Cu-ligand complex to render single-site Lys modification (**17b**).[112] Such a multicomponent approach extends to a metal-free phospha-Mannich reaction (**17c**, Fig. 5) as well.[113] Later, sulfonyl acrylates

(**17d**, Fig. 5)[114] and semi-oxamide vinylogous thioesters (**17e**, Fig. 5)[115] also delivered site-selective lysine modification.

Residue-specific labelling

The N-terminal residue and N^{α}-NH_2 can create a unique combination to bypass site-selectivity and deliver residue-specific modification of proteins. For example, the N-Cys forms an imine that renders thiazolidine through an intramolecular reaction (Fig. 6a).[116] The aldehyde used in this process can be further equipped with boronic acid to stabilize thiazolidine through a B-N dative bond (Fig. 6b).[117] This reagent was further functionalized with the ester to drive acyl transfer after stabilized thiazolidine bioconjugation (Fig. 6c).[118] In contrast to all the proteinogenic amino acids, the N-Gly offers no side chain residue. This attribute enabled an o-substituted aromatic aldehyde to generate a latent nucleophile (Fig. 6d).[119] Subsequently, it reacts with an aldehyde to deliver the N-Gly-specific aminoalcohol. The N-Pro offers another unique possibility in the form of a secondary amine. It allowed its selective modification by oxidative coupling with aminophenols.[120] In another example, the N-Pro forms iminium ions with aldehyde, which rapidly react with nucleophilic boronates to produce proline bioconjugates through the Petasis reaction (Fig. 6e).[121]

Modular methods

As we noticed in the discussed sections, the inherent reactivity and solvent accessibility define the reactivity hotspot or order for nucleophilic residues in bioconjugation. Going beyond the protein-defined reactivity order for modular single-site modification of proteins presents a highly daunting task. The drug discovery efforts to expand the druggable proteome and the evolution of discovery chemoproteomics offered the initial insights. It became evident that proximity control could be a powerful tool to supersede the reactivity preferences of proteinogenic residues toward an electrophilic system. In the past decade, ligand-directed (Fig. 7a)[122] and linchpin-directed modification (Fig. 7b)[123] offered comprehensive platforms to meet this challenge. For the first case, the ligand-protein interaction regulates the selectivity; hence modularity requires a new ligand. On the contrary, the latter initiates with a chemoselective step and ends with an irreversible site-selective reaction, while reagent design allows control over modularity.

Ligand directed modification: The advancement of chemoproteomics for covalent inhibitor or interactome identification fuelled the discovery of ligands to target enzymes (Fig. 7a).[124] Tethering a non-cleavable electrophile with ligand enabled selective residue modification in the binding domain. It assisted in targeted covalent inhibitor development in the last two decades.[125] Later, this approach enabled single-site modification of an endogenous protein.[126] Here, the ligand localizes the cleavable electrophile near a nucleophilic residue for selective modification without perturbing the protein function. The continuing efforts have strengthened this approach for selective protein modification.[127-134]

Linchpin directed modification: The linchpin-directed modification (LDM®) renders simultaneous control over reactivity, chemoselectivity, site-selectivity, and modularity.[135] At first, the LDM reagent (F_K-spacer-F_X) reacts rapidly with multiple Lys residues using F_K handle to create linchpins and regulate proximity (Fig. 7b). The design of the spacer defines the placement of the second handle (F_X). The selection of F_X and its chemoselectivity regulates the residue

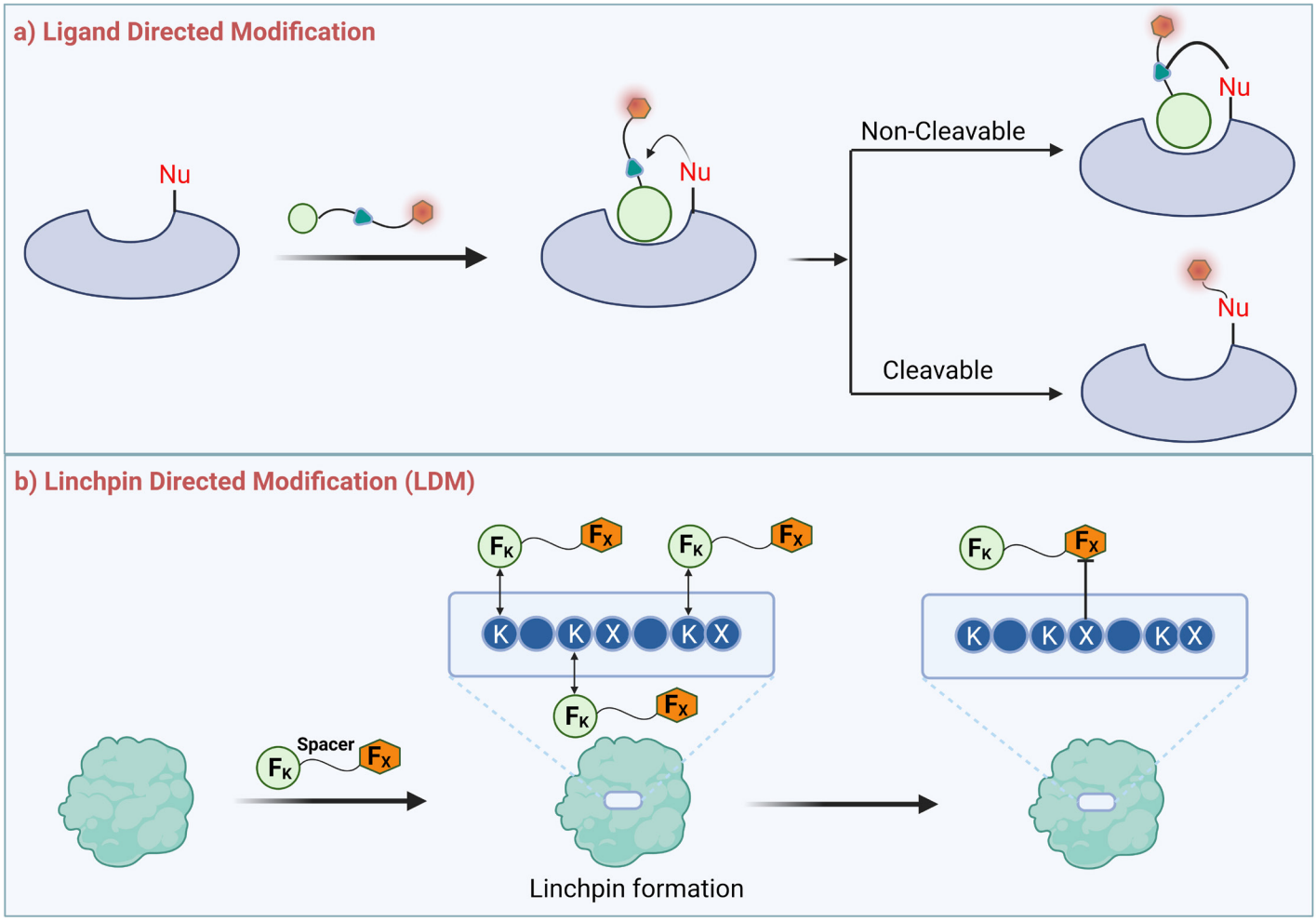

Figure 7. Modular methods: a) ligand directed modification and b) linchpin-directed modification

selection for modification, e.g., His, Lys, and Asp. The method translates to structurally diverse proteins and provides access to homogeneous antibody conjugates. F_K also enables the late-stage installation of tags, ordered immobilization, and metal-free protein purification to render analytically pure bioconjugates.

Conclusions and outlook

The technological demand for highly selective protein modification remained largely unmet until recently. However, the last decade has witnessed a rapidly growing knowledge of chemistry principles and theories from this perspective. The increasing clarity on reactivity and selectivity landscape with proteins has helped the community to accelerate the method development. The cumulative impact makes it now possible to control diverse selectivity attributes along with the reactivity and render precisely engineered bioconjugate. However, there are multiple unsolved challenges in the field. It requires electrophiles or reactive intermediates that can offer unique reactivity profiles for a more significant segment of proteinogenic residues. The type of bond constructs and their subsequent stability spectrum are still limited. The exclusive site-selectivity, homogeneity, and batch-to-batch consistency remain extremely challenging with complex substrates such as antibodies, virus-like particles, and viruses.

The exponential rise of biologics such as therapeutic proteins, antibody-based drugs, and conjugate vaccines has contributed to the increased investment in this field of research. In parallel, the last couple of years have seen an overlap between bioconjugation and chemoproteomics-driven development of covalent inhibitors. This bridge will grow stronger with time to benefit both segments. Still, in the early stages, the field has witnessed increased participation from multiple countries. The growing community promises to present solutions to the pre-existing technological demands while creating new avenues.

Acknowledgements

Our research is supported by SERB, DST, DBT, SERB-PACE (IPA/2021/000148), and IISER Bhopal. R.V., M.K., R.M., K.T. are recipients of a research fellowship from CSIR, PMRF and IISER Bhopal. P.C. is a recipient of postdoctoral fellowship from SERB-PACE. V.R. is a recipient of the Swarnajayanti Fellowship (DST/SJF/CSA-01/2018-19 and SB/SJF/2019-20/01). Chemoffice and Biorender were used for graphical content.

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