

## ADVANCED REVIEW



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# Noncovalent PEGylation of protein and peptide therapeutics

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## Abstract

Clinical applications of protein therapeutics—an advanced generation of drugs characterized by high biological specificity—are rapidly expanding. However, their development is often impeded by unfavorable pharmacokinetic profiles and largely relies on the use of drug delivery systems to prolong their in vivo half-life and suppress undesirable immunogenicity. Although a commercially established PEGylation technology based on protein conjugation with poly(ethylene glycol) (PEG)—protective steric shield resolves some of the challenges, the search for alternatives continues. Noncovalent PEGylation, which mainly relies on multivalent (cooperative) interactions and high affinity (host–guest) complexes formed between protein and PEG offers a number of potential advantages. Among them are dynamic or reversible protection of the protein with minimal loss of biological activity, drastically lower manufacturing costs, “mix-and-match” formulations approaches, and expanded scope of PEGylation targets. While a great number of innovative chemical approaches have been proposed in recent years, the ability to effectively control the stability of noncovalently assembled protein–PEG complexes under physiological conditions presents a serious challenge for the commercial development of the technology. In an attempt to identify critical factors affecting pharmacological behavior of noncovalently linked complexes, this Review follows a hierarchical analysis of various experimental techniques and resulting supramolecular architectures. The importance of in vivo administration routes, degradation patterns of PEGylating agents, and a multitude of potential exchange reactions with constituents of physiological compartments are highlighted.

This article is categorized under:

Therapeutic Approaches and Drug Discovery > Emerging Technologies  
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Disease

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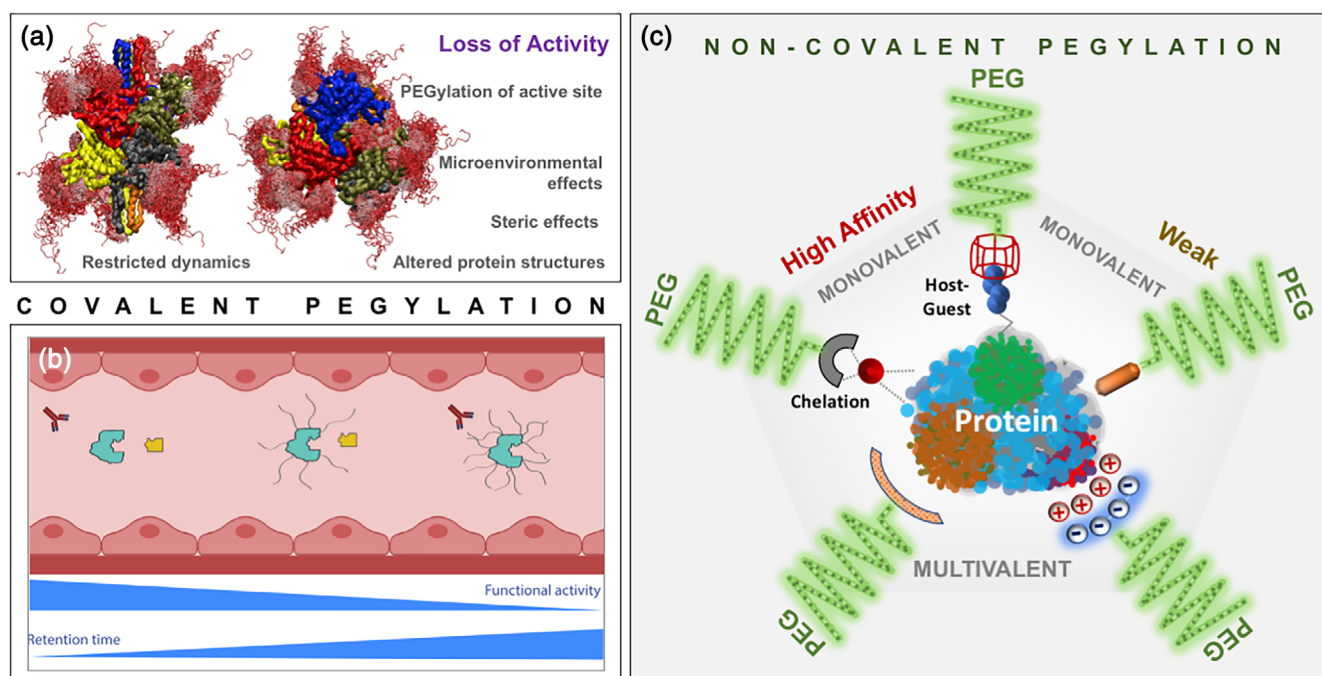
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## KEYWORDS

drug-delivery systems, host-guest complexes, multivalent interactions, noncovalent PEGylation, protein therapeutics

## 1 | INTRODUCTION

Macromolecular drugs, such as proteins and nucleic acids, are an increasingly important class of therapies (Ecker et al., 2015; Sliwowski & Mellman, 2013; Vaishya et al., 2015). Despite the unique advantages of therapeutic macromolecules, their development is frequently impeded by their suboptimal pharmacokinetic profiles, in particular short in vivo half-life and undesirable intrinsic immunogenicity (Bruno et al., 2013; Vaishya et al., 2015). PEGylation—a technology, which is based on covalent modification of proteins with a synthetic polymer, polyethylene glycol (PEG)—is currently one of the most successful techniques addressing these shortcomings (Morar et al., 2006; Sanchez Armengol et al., 2022; Swierczewska et al., 2015; Turecek et al., 2016; Veronese & Harris, 2002; Veronese & Pasut, 2005). Hydrophilic PEG forms a highly hydrated and dynamic steric barrier around the protein, which shields immunogenic sites on its surface from the body's immune system thereby introducing stealth characteristics (Caliceti & Veronese, 2003; Tabrizi et al., 2006). The PEG shield also increases the hydrodynamic volume of the protein, resulting in a reduced renal clearance (Caliceti & Veronese, 2003; Chertok et al., 2013; Duncan, 2003). Although PEGylation has been proven efficient in prolonging in vivo half-life and has achieved commercial success, the technology still suffers from limitations. In particular, covalently bound PEG chains can block active sites of the protein resulting in significant reduction or even loss of therapeutic efficacy (Figure 1a,b; Mero et al., 2011; Shi et al., 2022; Zaghmi et al., 2019). Also, covalently PEGylated proteins require expensive multistep purifications from reaction byproducts, which dramatically (10-fold and more) increases cost of the drug (Fee & Van Alstine, 2006; Mero et al., 2011; Mueller et al., 2011b). These shortcomings have intensified the quest for alternative “noncovalent” or supramolecular approaches, which can broaden the scope of protein drug candidates and drastically reduce production costs. It has also been suggested that noncovalent



**FIGURE 1** (a) Simulated structure of covalently PEGylated enzyme (glutamate dehydrogenase) and potential reasons for the observed loss of activity. Adapted with permission from (Zaghmi et al., 2019). Copyright © 2019. Elsevier Ltd. (b) Covalent PEGylation reduces interaction with opsonic molecules but may affect the intended biological activities of modified protein drug. Adapted with permission from Shi et al. (2022). Copyright © 2021. Elsevier B.V. (c) Various approaches explored for noncovalent PEGylation.

PEGylation can reduce the risk of immunogenic response and simplify regulatory approval, as the PEG chain is only transiently associated with the protein (Webber, Appel, Vinciguerra, et al., 2016).

The concept of supramolecular PEGylation is not limited to a temporary protection of protein with subsequent release of the drug in an approach similar to releasable covalent PEGylation (Filpula & Zhao, 2008) or other modulated drug release nanotechnologies (Mitchell et al., 2021). It embraces the idea of protection, in which protein modification is dynamic in nature and is based on various host–guest or polyelectrolyte exchange reactions (Dam & Brewer, 2008; Maikawa et al., 2021; Turnbull, 2011; Webber & Langer, 2017). Noncovalent PEGylation, which avoids site-specific attachment of PEG chains, envisions at least periodic exposure of the active site of the protein and more effective modulation of pharmacokinetics. A great number of experimental techniques have been developed with the above objectives. They include monovalent and multivalent approaches and utilize a variety of high-affinity interactions, such as host–guest complexation and chelation, as well as ionic bonds and hydrophobic association (Figure 1c).

An excellent recent minireview on noncovalent PEGylation, which highlighted the overall significance of the approach, was primarily focused on studies related to multivalent interactions between proteins and polymers (Reichert & Borchard, 2016). The present review provides a hierarchical analysis of various approaches to noncovalent PEGylation, which further extends to rapidly evolving clinically oriented technologies on the basis of monovalent high-affinity complexation, as well as weakly bound single-attachment systems, which may offer advantages for improving shelf-life of pharmaceutical formulations. Special attention is given to fundamental relationships between the affinity of *in vitro* interactions, physiological stability of the resulting PEGylated complexes and the recently emphasized critical role of *in vivo* administration routes. Finally, whenever possible, various supramolecular architectures and their formulations are benchmarked against the corresponding covalently linked counterparts, to critically evaluate the potential of the technology against the unmet needs of life sciences applications.

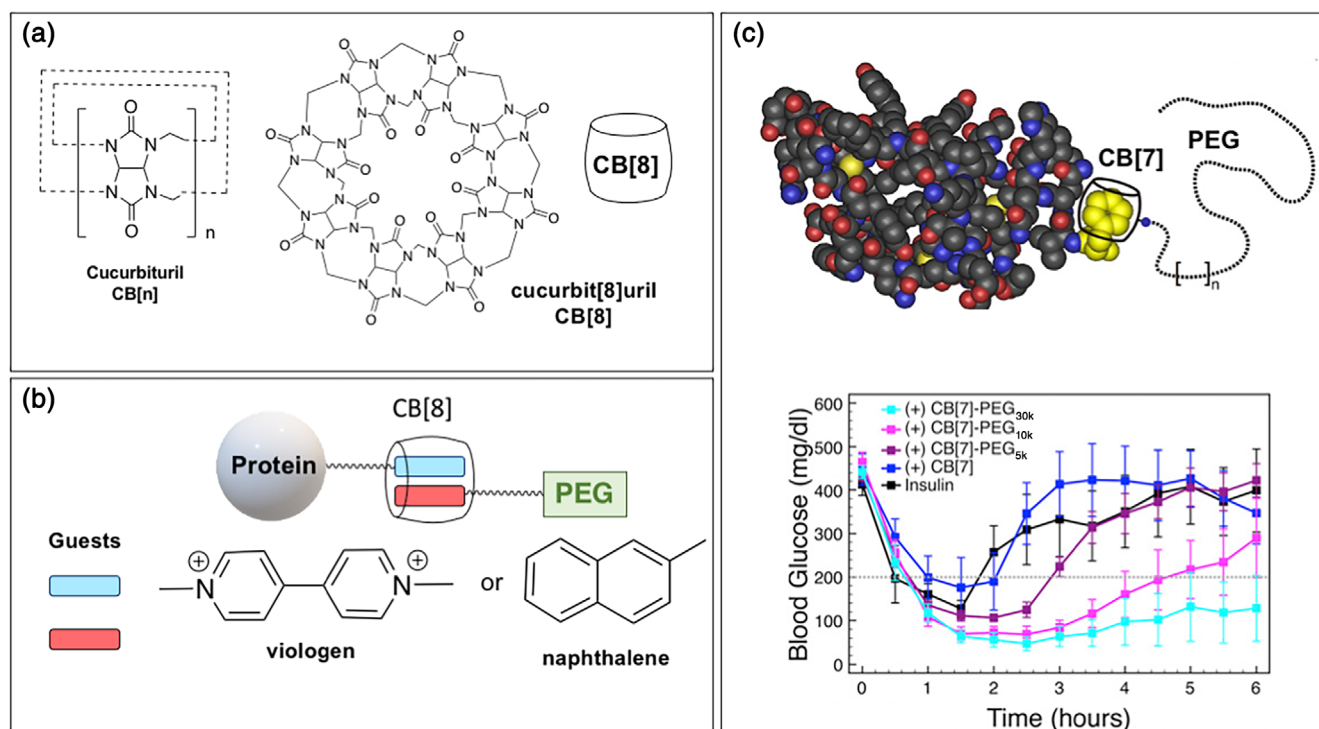
## 2 | PEGYLATION VIA MONOVALENT INTERACTIONS

The concept of single-point attachment of functionalized PEG to the protein surface essentially mirrors the approach of conventional PEGylation technology. However, the quest for the appropriate type of noncovalent linkage remains the main challenge on the way to its successful clinical development. With few exceptions, PEGylated proteins appear to be highly susceptible to the effects of physiological environment, such as high-ionic strength and protein exchange reactions, and only rarely display required stability *in vivo*.

### 2.1 | High-affinity host–guest interactions

One of the most promising monovalent modification technologies is based on high-affinity binding offered by host–guest complexation. The approach is generally limited to proteins containing N-terminal aromatic residues, or it requires chemical modification of the protein. In the latter case, a small molecule recognition moiety needs to be installed on the protein surface, although such treatment may still be preferred to reactions involving end-functionalized PEG macromolecules. PEGylation using host–guest interaction is also frequently referred to as a “reversible” or “dynamic” PEGylation, which generally implies that the protein can be released under certain conditions without chemical breakdown of covalent bonds (Biedermann et al., 2011).

Host–guest-complex mediated PEG–protein binding can be viewed as linking the components through a supramolecular “handcuff.” The cucurbiturils—macrocylic molecules made of glycoluril monomers linked by methylene bridges (Figure 2a)—exhibit exceptional binding affinities to a broad range of guest molecules (Braegelman & Webber, 2019; Hou et al., 2017; Wang et al., 2022). In particular, the ability of cucurbit[8]uril (CB[8]) to accommodate up to two organic guest molecules simultaneously was used to provide for specific binding interactions between the complementary labeled PEG polymer and bovine serum albumin (BSA; Biedermann et al., 2011). Both, the protein BSA and PEG were functionalized with either viologen or naphthalene for the formation of CB[8] ternary complex (Figure 2b). It was found that strong and specific binding between the complementarily modified PEG and BSA only occurred in the presence of the host CB[8], but not in the absence of one of the three critical components. However, no biophysical or *in vivo* assessment of the complex was conducted in this study to evaluate its stability under physiological conditions.



**FIGURE 2** (a) Chemical structures of cucurbiturils (CB[n]). (b) Schematic presentation of CB[8] mediated PEGylation of protein using viologen and naphthalene modified components as guest moieties. (c) CB[7] mediated PEGylation of insulin via its N-terminal aromatic phenoxy group and blood glucose levels in diabetic mice demonstrating extended activity of CB[7]-PEG modified insulin with high-molecular weight derivatives showing best results (the dotted gray line shows the standard criteria for normoglycemic level in diabetic mice). Adapted with permission from Webber, Appel, Vinciguerra, et al. (2016).

As mentioned above, no chemical modification is needed for proteins containing N-terminal phenoxy groups, as cucurbiturils are capable of forming complexes with terminal tryptophan or phenylalanine groups with a dissociation constant in a micromolar range (Webber, Appel, Vinciguerra, et al., 2016). To that end, CB[7] end-modified PEGs were successfully employed for cucurbituril mediated PEGylation of insulin (Figure 2c). The high-affinity interactions between protein and PEG in such complexes provided for sufficient physiological stability in diabetic mice, thereby enabling beneficial modulation of pharmacological profiles of the drug in the polymer molecular weight-dependent manner (Figure 2c). Further research demonstrated that formulations of insulin, modified with CB[7]-PEG exhibited over 10-fold extended stability compared with commercial formulations of insulin (Maikawa, Smith, Zou, Meis, et al., 2020). Moreover, simultaneous noncovalent PEGylation of insulin and a small peptide, pramlintide provided for superior stability and enabled their co-formulation at a physiological pH (Maikawa, Smith, Zou, Roth, et al., 2020). In diabetic rats, this dual formulation drastically improved the pharmacokinetic profile compared with separate administration of the two hormones. Further studies explored the effect of N-terminal modification of insulin, which allowed pH-tunable supramolecular PEGylation (Meudom et al., 2023). This technique was used to enhance formulation stability without impacting the precision of blood glucose control via excessive subcutaneous depot retention. The CB[7]-mediated PEGylation approach was also extended to proteins lacking N-terminal aromatic amino acid guests: glucagon and anti-CD20 antibody—a variant of Rituximab. Although the binding was expected to be somewhat lower in that case, the noncovalently bound conjugates still exhibited a stabilizing effect when compared with native proteins (Webber, Appel, Vinciguerra, et al., 2016).

An important recent study compared stability and in vivo activity of CB[7] bound insulin-PEG complexes, in which the host moiety was attached (a) to PEG allowing its interaction with N-terminal group as described above or (b) to insulin, essentially “handcuffing” it to adamantane or xylylenediamine modified PEG (Maikawa et al., 2021). Authors found that the latter, CB[7] insulin modification method, resulted in a greatly superior stability of complexes. Interestingly, intravenous administration of formulations in diabetic rats showed no change in insulin half-life, indicating rapid dissociation of the complex upon dilution in the blood. However, when administered subcutaneously, higher binding

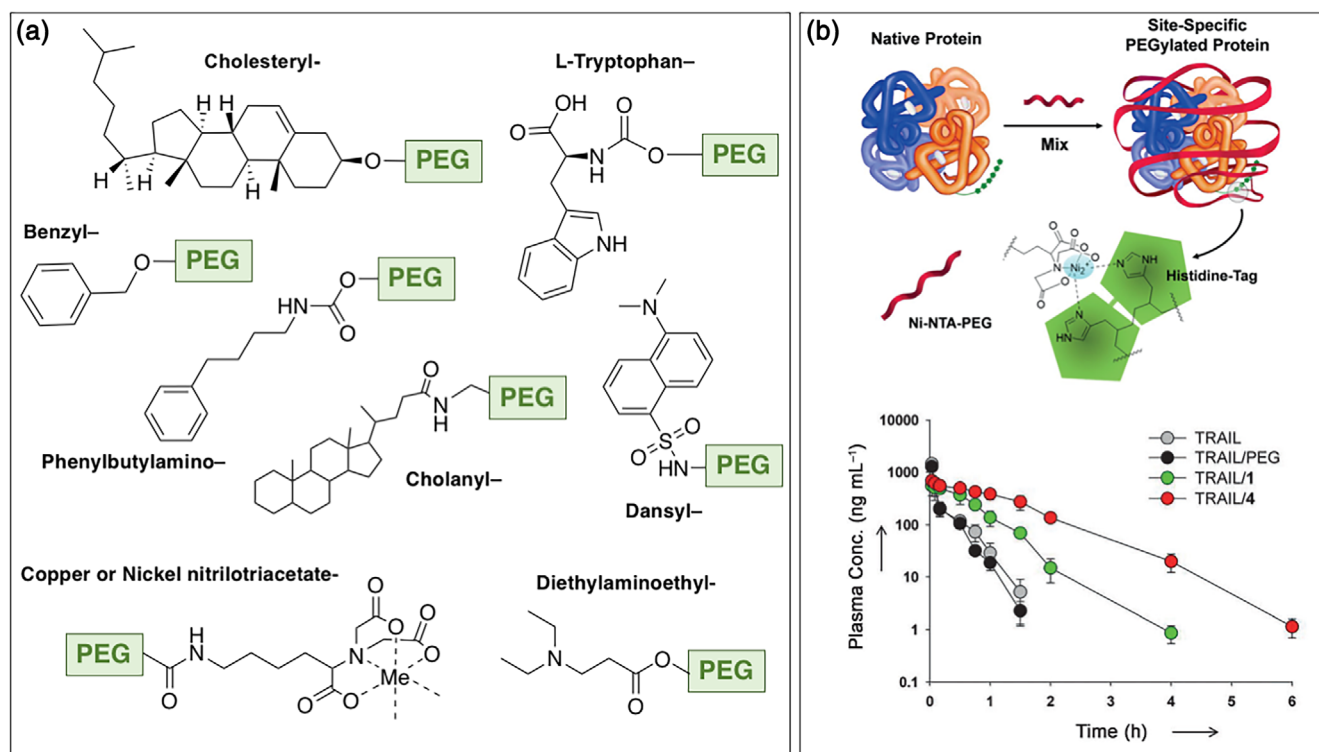


affinities in CB[7] mediated complexes corresponded to slower absorption of insulin from the subcutaneous depot, thereby prolonging drug exposure in the blood (Maikawa et al., 2021).

Overall, the dynamic nature of host–guest mediated PEGylation may offer some advantages over covalent PEGylation in certain environments, such as subcutaneous administration, in which slow release of the drug appears to be essential. The chemistry of host–guest PEGylation continues to extend with other systems, such as  $\beta$ -cyclodextrin–adamantane (Hirotsu et al., 2017) or recently suggested anionic calixarenes (Mummidivarapu et al., 2018). However, the relatively narrow scope of proteins with N-terminal phenoxy groups or somewhat challenging methods of synthetic modification of PEG or proteins, may be considered as serious limitations of the approach.

## 2.2 | Hydrophobic association, ionic, and coordinate bonds

Most research conducted in the field appears to focus on the development of methods preventing protein aggregation in vitro and improving product shelf-life. Figure 3a shows some of the monofunctionalized PEGs used for those purposes. A series of studies described the synthesis of monofunctionalized polyethylene glycols containing phenylbutylamino-, benzyl-, cholesteryl- (Mueller et al., 2012), dansyl- (Mueller et al., 2011a), and L-tryptophan- (Mueller et al., 2011b) with the objective of stabilizing proteins, such as salmon calcitonin (sCT) and hen egg-white lysozyme (HEL), against aggregation. The formation of complexes was driven by a single site ionic bond or hydrophobic association. Both dansyl and tryptophan derivatives reduced the aggregation of sCT for up to 70 h (Mueller et al., 2011a, 2011b). Cholesteryl functionalized PEG was able to completely suppress aggregation of HEL, whereas some deterioration of protein was observed when a benzyl-derivative of PEG was used. Phenylbutylamino-, tryptophan-, and dansyl- functionalized PEGs reportedly prolonged the lag phase of aggregation and reduced protein aggregation rate (Mueller et al., 2012). Authors also noted that while no beneficial effect of hydrophobically modified PEGs was observed on the stabilization of sCT, aggregation of HEL was progressively reduced with increased hydrophobicity of the headgroup.



**FIGURE 3** (a) Structures of PEGs functionalized with various end groups capable of noncovalent interactions with proteins; (b) schematic illustration of noncovalent PEGylation of a His-tagged protein (TRAIL) with the reactive Ni-NTA-PEG and pharmacokinetic profiles of TRAIL, covalently modified Trail (TRAIL/PEG), noncovalently modified TRAIL with mono (TRAIL/1) and bifunctional (TRAIL/4) Ni-NTA-PEG. Adapted with permission from Kim et al. (2013). Copyright © 2013. Wiley-VCH Verlag GmbH & Co.

In an independent study, cholesteryl functionalized PEG was studied for noncovalent modification of insulin through mixing their aqueous solutions and subsequent lyophilization (Asayama et al., 2018). Noncovalent PEGylation, which was confirmed by gel electrophoresis and gel-filtration chromatography, was effective in protecting insulin against digestion by protease, but failed to prevent its recognition by monoclonal antibody. In mice, noncovalently PEGylated insulin was more effective in suppressing blood glucose level compared with native peptide (Asayama et al., 2018). It is worth noting that the results were limited to 4 h post-injection with 1-h data showing essentially similar glucose levels. The same authors reported noncovalent modification of catalase using diethylaminoethyl end modified PEG (Asayama et al., 2017). Such modification did not significantly affect enzymatic activity or secondary structure of the protein. The activity of catalase was protected in the presence of a protease, trypsin, or 10% fetal bovine serum.

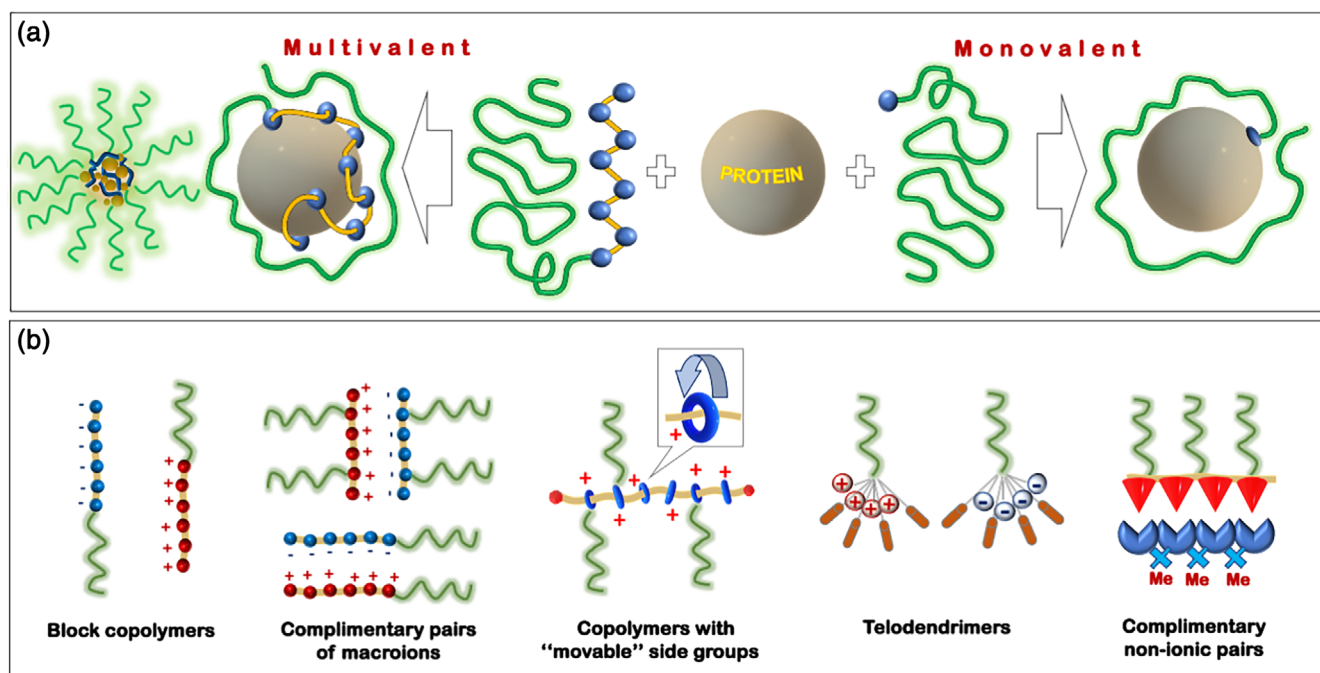
Self-aggregating cholanyl derivatives of PEG (Figure 3a) were studied for noncovalent PEGylation of recombinant human colony stimulating factor (rh-G-CSF; Salmaso et al., 2012). Authors emphasized the self-assembly of monofunctionalized PEGs into micelles of nano-scale dimensions with polycyclic moieties oriented toward their core. Protein binding to micelles caused some structural changes in rh-G-CSF; however, this did not impair biological activity of the cytokine. In vivo studies in rats, which included both intravenous and subcutaneous administration, demonstrated the ability of micelle mediated PEGylation to prolong the half-life of rh-G-CSF. This was especially pronounced after subcutaneous administration, which resulted in up to 12-fold prolongation with pharmacokinetic behavior similar to that of covalently PEGylated cytokine (Salmaso et al., 2012).

PEGs, which were functionalized with copper nitrilotriacetate (Cu-NTA) end groups—a chelating agent capable of forming coordination bonds with histidine (Figure 3a), were studied for their interactions with hemoglobin, human growth hormone (hGH), granulocyte colony stimulating factor (G-CSF), interferon  $\alpha$ -2b (IFN), and insulin (INS) (Mero et al., 2011). Surface plasmon resonance studies demonstrated that only eight-arm PEG, which contained eight Cu-NTA moieties on flexible PEG arms, associated strongly with proteins and especially with G-CSF (dissociation constant in nanomolar range). However, even those complexes failed to improve the half-life of the protein when tested in Sprague–Dawley rats (Mero et al., 2011).

In contrast, similar approach, which utilized Ni-NTA derivatized PEG and tumor necrosis factor-related apoptosis inducing ligand (TRAIL) with an oligo-histidine tag (His-tag) (Figure 3b), produced impressive in vivo results (Kim et al., 2013). In their study, authors compared TRAIL, which was noncovalently modified with PEG containing either one or two Ni-NTA moieties (TRAIL/1 and TRAIL/4, respectively), with covalently PEGylated and native protein. After intravenous injection in rats, noncovalently modified TRAIL/4 showed a prolonged half-life compared with both covalently PEGylated protein and native TRAIL (Figure 3b) and its bioavailability was enhanced by almost four-fold when compared with unmodified protein. Authors also note that PEG with two Ni-NTA arms showed longer half-life and over two-fold increase in bioavailability compared with its single-arm counterpart. Moreover, TRAIL/4 was the only drug which prevented tumor regrowth in human colon cancer HCT116-bearing mice and maintained the highest tumor growth inhibition value (52%) throughout the study. Collectively, noncovalent PEGylation demonstrated three- to four-fold improved efficacy over native protein in terms of solution stability, in vivo half-life, and bioavailability.

### 3 | MULTIVALENT INTERACTIONS

Efforts on the search for noncovalent protein–PEG links, which can provide for adequate complex stability under physiological conditions, have been extended to the development of macromolecular binding agents that offer an advantage of multivalent interactions (Figure 4a). Multivalent or polyvalent interactions have long been recognized for their potential to achieve superior binding between biological partners (Mammen et al., 1998; Matsarskaia et al., 2020; Turnbull, 2011) and spontaneous assembly of charged synthetic macromolecules with proteins have been also extensively researched (Gao et al., 2019; Horn et al., 2019). It has been established that protein–polyelectrolyte interactions are favored either by entropy changes due to counterion release, or by the ability of flexible macroions to find oppositely charged surfaces on the protein—charge anisotropy model of interactions (Gao et al., 2019; Horn et al., 2019; Kayitmazer et al., 2013; Xu et al., 2011). Moreover, protein complexes with highly charged and flexible polyp-hosphazene polyelectrolytes are physiologically stable and demonstrated their utility in a clinical environment (Andrianov & Langer, 2021). It has also been reported that some multivalent interactions may involve a “bind and slide” mechanism, in which a counter partner may move from one binding site to another along a polymer chain (Dam & Brewer, 2008; Turnbull, 2011). It can be envisioned that the latter can further advance the development of



**FIGURE 4** (a) Comparison of multivalent and monovalent attachment of PEG chains; (b) schematic presentation of different types of multivalent attachment pathways.

dynamic PEGylation, in which the active site of the protein can be made periodically available for interaction with its biological target without the protein being released from the complex.

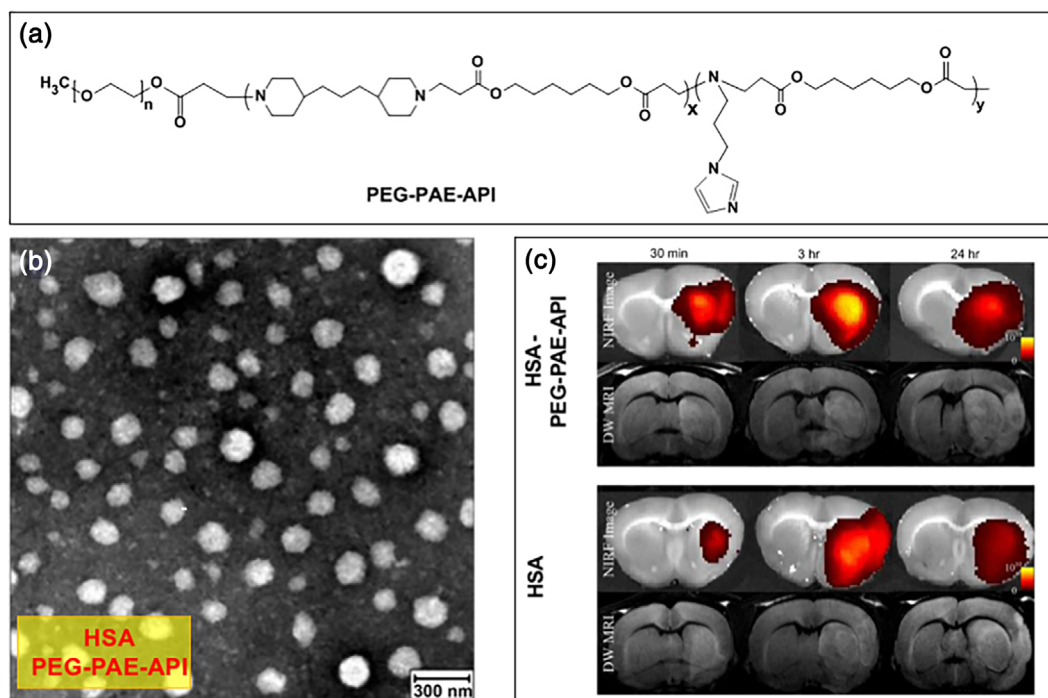
### 3.1 | PEGylated block copolymers

Block and graft copolymers containing PEG and ionic polymer domains have been the most frequent choices for noncovalent PEGylation of proteins and peptides (Figure 4b). The spontaneous self-assembly of poly(ethylene glycol)-*block*-poly(aspartic acid) with hen egg white lysozyme (HEL) into stable core-shell type of nano-assemblies was first reported over two decades ago (Harada & Kataoka, 1998). The cooperativity manner of interactions was established and it was estimated that 36 molecules of lysozyme were packed with 42 chains of block copolymer in the condensed core of micelle-like complexes. It was also found that protein-copolymer molar ratio did not affect the association of HEL and the core size, but influenced the thickness of the corona (Harada & Kataoka, 1999a). The enzymatic activity of PEGylated HEL against *Micrococcus luteus* cells was completely inhibited, but completely recovered upon addition of sodium chloride. Furthermore, when low molecular mass substrate was used instead of cells, PEGylated enzyme was more active compared with unbound enzyme—an indication of change in substrate binding specificity (Harada & Kataoka, 2001a, 2001b). Overall, high salt-sensitivity of self-assembly in the system and the reversible nature of complex formation was reported (Harada & Kataoka, 1999b). The control over complex dissociation and related change in enzymatic activity was also achieved using a pulse electric field (Harada & Kataoka, 2003). Such on-off control of enzymatic activity presented interest in terms of engineering of an intelligent bioreactor, but demonstrated complex instability at physiological salt concentrations, which could limit the utility of the system for drug delivery applications.

To overcome this issue, the authors used glutaraldehyde to cross-link polymer-trypsin complexes, which resulted in active and stable assemblies (Jaturanpinyo et al., 2004). When this approach was applied to HEL, the resulting glutaraldehyde cross-linked complexes showed excellent stability against ionic strength increase, dilution, pH variation, and addition of dioxan (Yuan et al., 2005). However, the authors note that toxicity of glutaraldehyde and the irreversibility of the cross-linking may present further challenges for clinical development of such complexes (Lee et al., 2009). In an unrelated study, the instability of HEL complexes with PEGylated polyacid under physiological salt concentrations was addressed via ionic cross-linking of carboxylic acid moieties of the polymer with a physiologically benign cationic molecule, spermine (Andrianov et al., 2020).

A different approach to improving stability of protein–polymer complexes involved charge conversion of the protein (Lee et al., 2009). Cytochrome C, an essential protein in the electron transfer of the mitochondria, was modified with citraconic anhydride and cis-aconitic anhydride to increase the density of negative charges and then complexed with cationic block copolymer, PEG–poly[*N*-(*N'*-(2-aminoethyl)-2-aminoethyl)aspartamide] (Lee et al., 2009). The stability of the complex under physiological salt conditions was significantly improved and its efficiency in intracellular delivery of cytochrome C to human hepatoma cells was demonstrated. This charge-conversional methodology was also successfully applied to the delivery of IgG antibody into the cytoplasm of human hepatoma cells (Lee et al., 2010). A systemic study on chemical “supercharging” of proteins with succinic anhydride was conducted using four model proteins: α-chymotrypsinogen, lysozyme, myoglobin, and ribonuclease (RNase A) (Obermeyer et al., 2016). These proteins were co-assembled with block copolymer containing graft oligo(ethylene glycol) chains, which was synthesized by polymerizing 4-vinylpyridine with oligo(ethylene glycol) methyl ether methacrylate and subsequent quaternization of amino groups. Overall, it was found that increasing the charge density on the protein improved the stability of the complexes to salts (Obermeyer et al., 2016).

Block-copolymers containing poly(amino acids) domains have also been extensively studied (Nieto-Orellana et al., 2017; Pippa et al., 2015; Tsiourvas et al., 2012). Copolymers containing 750 Da and 2 kDa PEG and positively charged oligolysine or oligoarginine were demonstrated to interact with insulin under physiological conditions (Tsiourvas et al., 2012). Optimal protection against enzymatic degradation by trypsin and α-chymotrypsin was observed for the complex containing block copolymer of oligoarginine and high-molecular mass PEG. The half-release of insulin from the complex in simulated intestinal fluid was in the range of 1.5–2 h and the percent of released peptide was greater for a complex with high-molar mass PEG chains. The proof of complex formation between block copolymer of poly(L-lysine) and higher molar mass (5 kDa) PEG was obtained by dynamic light scattering (Pippa et al., 2015). A pronounced increase in the hydrodynamic radius of the copolymer upon addition of insulin was interpreted by the authors as an enabling of stealth properties, which can potentially affect clearance properties in vivo. The effect of polymer topology on complex formation with protein was studied using block copolymers of PEG and poly(glutamic acid) (Nieto-Orellana et al., 2017). Copolymers of two different architectures, linear and miktoarm star, which contained three poly(amino acids) and one 2 kDa PEG arms, were studied for interactions with lysozyme. It was found that linear



**FIGURE 5** (a) PEG-PAE-API block copolymer, (b) transmission electron microscopy (TEM) image of HSA/PEG-PAE-API complex, and (c) in vivo diffusion-weighted magnetic resonance imaging MRI (DW-MRI), near-infrared fluorescence (NIRF) images of rat brains in animals injected with fluorescently labeled HSA complexed with PEG-PAE-API and the protein alone. Adapted with permission from Gao et al. (2012). Copyright © 2012. Elsevier Ltd.



macromolecules were more efficient in sequestration of lysozyme than their star-shaped analogs with the same number of glutamic acid groups. The complexation resulted in the formation of complexes with hydrodynamic diameters in the 50–200 nm range with suppressed enzymatic activity compared with the unbound protein. However, this activity was recovered when the complex dissociated upon addition of poly(allyl amine) indicating the reversible character of complexation.

Physiologically stable PEGylated complexes of the model protein—human serum albumin, were assembled with a copolymer containing PEG and cationic poly( $\beta$ -amino ester) block (PEG-PAE-API), which was synthesized using Michael-type step polymerization of hexanediol diacrylate, trimethylenedipiperidine, and aminopropylimidazole with monofunctional PEG (Figure 5a) (Gao et al., 2012). The albumin-containing micelle with 66-nm diameter was stable under physiological pH and salt concentration (Figure 5b) and was tested for protein cargo delivery to acidic pathologic areas in rat model of cerebral ischemia. When injected intravenously, the accumulation of fluorescently labeled PEGylated protein in the ischemic tissue of the brain was significantly higher at 30 min and 3 h than that of the protein itself (Figure 5c; Gao et al., 2012).

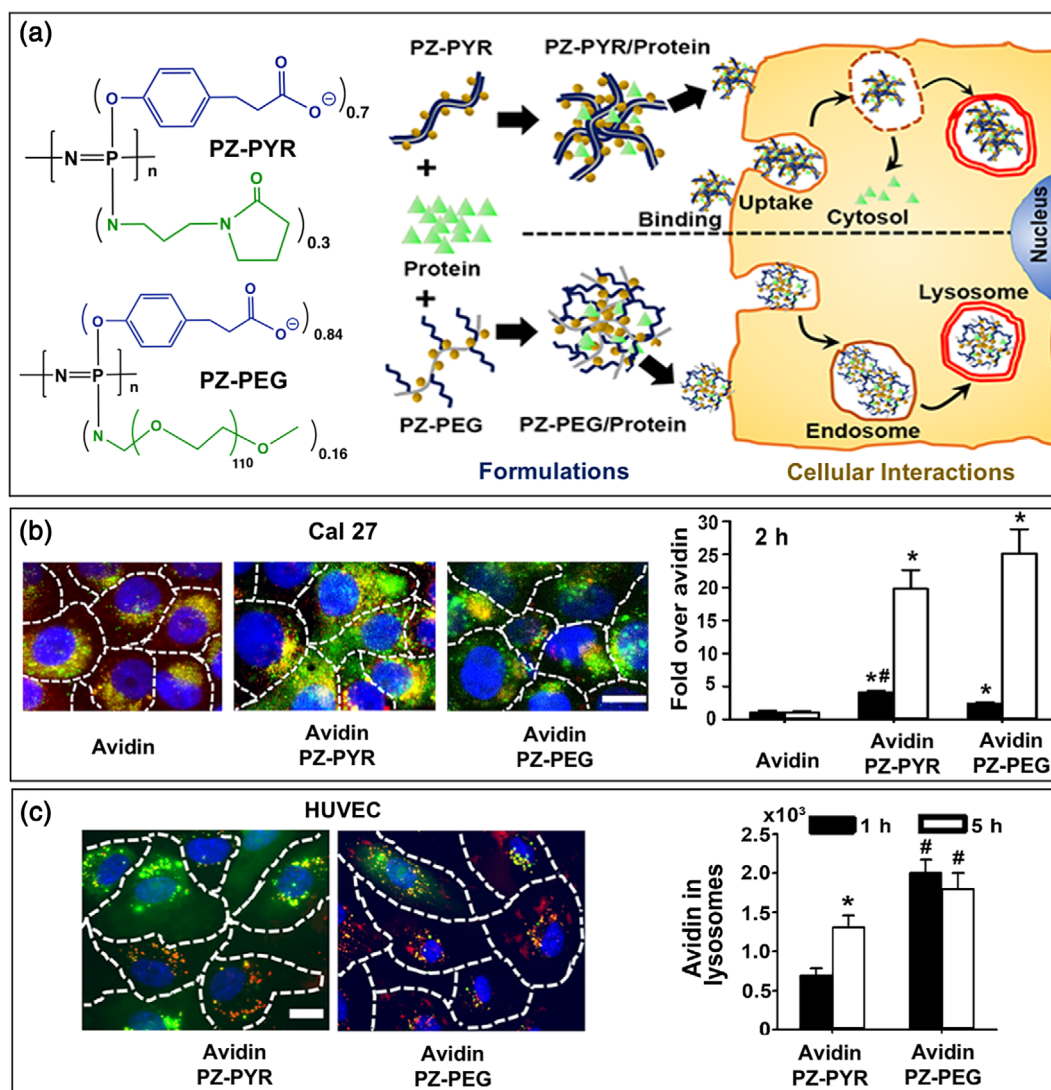
A block copolymer of PEG and poly(*N,N*-dimethylaminoethyl methacrylate), PEG-*b*-PAMA, was explored as an agent for noncovalent PEGylation of an important therapeutic enzyme—L-asparaginase (Kurinomaru & Shiraki, 2015). The formation of water-soluble complexes was detected by an increase in hydrodynamic diameter from 11 nm for the enzyme to 22 nm for its formulation with the polymer (Kurinomaru & Shiraki, 2015). No loss of secondary structure or decrease in enzymatic activity was observed. Authors noted that such noncovalent PEGylation successfully protected the enzyme against trypsin digestion and shaking-induced aggregation and that the stabilizing effects were similar to that of covalently PEGylated L-asparaginase. To improve stability of noncovalently PEGylated enzymes at high-ionic strength, PEG-*b*-PAMA was quaternized and studied for interactions with  $\alpha$ -amylase. The results of enzyme inhibition, which the authors considered as a marker of complex formation, indicated that only quaternization of polymer with the most hydrophobic benzyl groups allowed for complex formation under physiological conditions (Kuwada et al., 2016). The molecular dynamic simulation study revealed that PEGylation of  $\alpha$ -amylase using PEG-*b*-PAMA occurred through nonspecifically binding to the protein surface other than the active site, which caused noncompetitive inhibition of enzymatic activity (Kurinomaru et al., 2017).

A cationic block copolymer—poly(2-methyl-vinyl-pyridinium)-*b*-poly(ethylene-oxide)—was used for complexation with enhanced green fluorescent protein, which resulted in the formation of complex coacervate core micelles (Nolles, Westphal, et al., 2017; Nolles et al., 2015). It was found that a high-protein packaging density (450 protein copies in 68 nm diameter micelles) resulted in a restricted orientational freedom of the encapsulated molecules, influencing structural and spectral properties of the protein (Nolles, van Dongen, et al., 2017). The same polymer was used to encapsulate a number of other fluorescent proteins, which resulted in significant variations in encapsulation efficiency and explained by differences in charge distribution and structural parameters of the protein cargo (Nolles, van Dongen, et al., 2017). Stability studies utilizing encapsulated cyan, yellow, and blue fluorescent proteins pointed to the dynamic nature of complexes and emphasized the need of enhancing the rigidity and salt stability of these complexes (Nolles et al., 2018).

In studying interactions of a number of cationic and anionic polymers with enzymes, such as  $\alpha$ -chymotrypsin, authors noted the ability of polyelectrolytes to hyperactivate enzymes, increasing their activity by 7- to 18-fold (Kurinomaru et al., 2014). Although the mechanism of this effect is not sufficiently clear, it was suggested that ionic polymers may increase local concentration of the substrate, lower its activation energy, and stabilize the intermediate state in the catalytic reaction. A reversible character of polymer–protein interactions was also noted and on/off switching of enzyme activity mechanism was suggested on the basis of polyelectrolyte exchange reactions. To that end, inactivation of  $\alpha$ -amylase and  $\beta$ -galactosidase with PEG-*b*-PAMA can be reversed by adding poly(acrylic acid), which led to recovery of enzymes from the complex (Kurinomaru et al., 2012).

### 3.2 | PEGylated graft copolymers

Copolymers containing graft PEG chains have also been successfully employed for noncovalent PEGylation of proteins. A cationic comb-type polyelectrolyte—poly(ethylene glycol)-*graft*-poly(allyl amine) (PEG-*g*-PAA) was used to modify an anionic enzyme, *Aspergillus niger* glucose oxidase. Nano-scale (30 nm diameter) core–shell structured particles were formed, which were intended for a potential use in biosensors (Kawamura et al., 2008). The role of macromolecular architecture (molecular mass and density of graft PEG chains) was explored using complexation of two graft

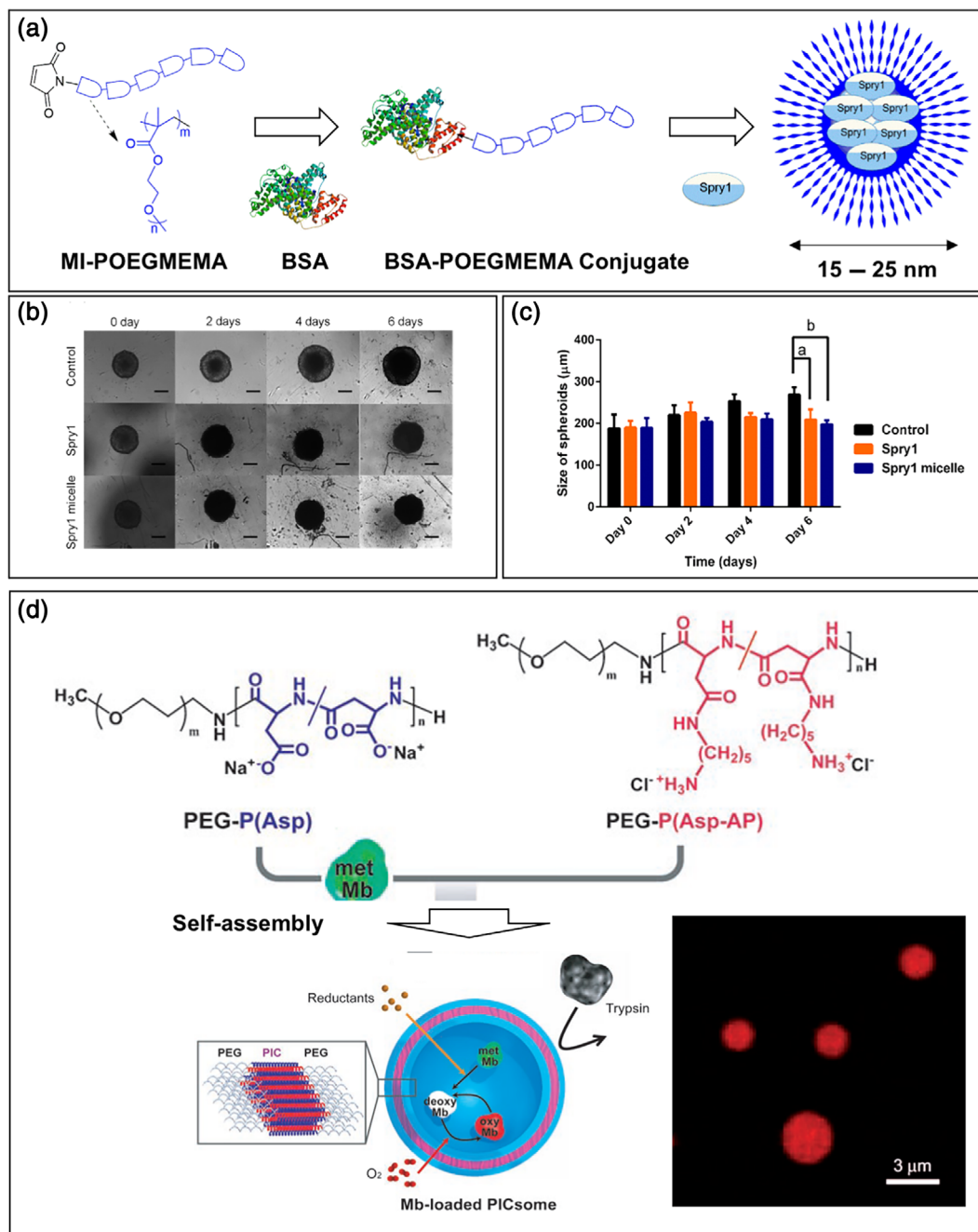


**FIGURE 6** (a) Chemical structures of PZ-PEG and PZ-PYR, their formulations with proteins and cellular pathways; (b) uptake of polymer complexes with avidin in Cal27 cancer cells (avidin on cell surface is in yellow, internalized—in green, blue—cell nuclei, dashed lines—cell borders as seen by bright field, scale bar 10  $\mu$ m, data are mean  $\pm$  SEM, \* $p$  < 0.05 relative to avidin, # $p$  < 0.05 comparing both polymers); (c) subcellular trafficking of polymer-avidin complexes in HUVEC cells—avidin in lysosomes (lysosomes—red, avidin in lysosomes—yellow, protein in vesicular compartments that are not lysosomes is in green, colocalization of FITC-avidin with lysosomes is expressed as the area of pixels colocalizing with lysosomes. Data are expressed as mean  $\pm$  SEM, \* $p$  < 0.05 relative to 1 h, # $p$  < 0.05 relative to PZ-PEG, dashed lines—cell borders as seen by bright field, scale bar = 10  $\mu$ m). Adapted with permission from Qamar et al. (2021). Copyright © 2022 by the authors.

copolymers (PEGylated pentosan polysulfate and dextran sulfate) with keratinocyte growth factor-2 (Khondee et al., 2011). It was found that protein binding was reduced for highly PEGylated polyanions compared with conjugates with fewer PEG chains and the molar mass of PEG grafts did not have any effect on protein binding. Authors note that the interactions were primarily enthalpy driven and the association constants were in the  $10^5$ – $10^7$   $M^{-1}$  range. Both polymers were able to stabilize the protein, as detected by an increase in its melting temperature.

Hydrolytically degradable graft copolymer, poly[di(carboxylatoethylphenoxy)phosphazene]-*graft*-poly(ethylene glycol), PZ-PEG, has been synthesized as a potential carrier for intracellular delivery of physiologically active protein cargo and was compared with a linear polymer containing the same type of anionic side groups and hydrophilic pyrrolidone moieties—poly[(carboxylatoethylphenoxy)(3-(2-oxo-1-pyrrolidinyl)propylamino)phosphazene] (Figure 6a; Martinez et al., 2017; Martinez et al., 2018; Qamar et al., 2021). Both polymers had high-binding avidity for a model protein—fluorescently labeled avidin, as well as physiologically active cargo Bax-BH3 peptide (apoptosis-inducing

agent) and anti-F-actin antibody (for revealing filamentous actin in the cytosol; Qamar et al., 2021). It was found that either polymer provided for trafficking through the intracellular endolysosomal route (Figure 6a,b). Mechanistically, complexes did not actively induce endocytosis, but piggybacked into the cell by any endocytic route the cell is using at the moment (Qamar et al., 2021). The ability of polyphosphazenes to attach to the cell membrane allowed them to enter the cell on the membrane at sites where endocytic vesicles are being formed when a cell performs endocytosis to uptake molecules from the cell medium or recycle its membrane components. Macromolecular architecture of



**FIGURE 7** (a) Conjugation of maleimide functionalized poly(oligo (ethylene glycol) methyl ether methacrylate) (MI-POEGMEMA) and BSA and formation of complex micelles with angiogenesis inhibitor Sprouty 1 (Spry1); (b) Breast carcinoma MCF-7 spheroids were treated with Spry1, POEGMEMA-BSA-Spry1 complex, and water as a control. The scale bar is 100  $\mu\text{m}$ . (c) Size variation of spheroids in each group with time. Data are expressed as mean  $\pm$  standard deviation,  $n = 3$ . (Statistical difference: (a)  $**p < 0.01$ ; (b)  $***p < 0.001$ ). (a–c) Adapted with permission from Jiang et al. (2016). Copyright © 2016. American Chemical Society. (d) Self-assembly of complexes using a pair of oppositely charged block ionomers with myoglobin (Mb) and its reversible oxygenation and cross-sectional image of complexes observed by confocal laser scanning microscopy. Adapted with permission from Kishimura et al. (2007). Copyright © 2007. John Wiley and Sons.

polyphosphazenes and their pH-dependent membrane disruptive activity allowed regulation of intracellular pathways (Figure 6a,c). Linear polymer structures offered endo-lysosomal escape without compromising cell viability and therefore enabled cytosolic delivery of active protein cargo, while graft topology favored protein retention in endo-lysosomal vesicles (Qamar et al., 2021).

### 3.3 | PEGylated proteins as macroreagents

Noncovalent PEGylation on the basis of protein–protein interactions was also explored (Jiang et al., 2016). A negatively charged bovine serum albumin (BSA) was covalently attached to poly(oligo (ethylene glycol) methyl ether methacrylate), POEGMEMA, and the resulting “macroreagent” was investigated for binding proteins with overall positive charge—lysozyme and angiogenesis inhibitor—Sprouty 1 (Figure 7a). Such “protein–protein”-mediated modification lowered the half maximal inhibitory concentration ( $IC_{50}$ ) value of Sprouty 1 agent against breast cancer cells. The high-anticancer efficacy of the complex was also verified by inhibiting the growth of three-dimensional MCF-7 multicellular tumor spheroids (Figure 7b,c). Although the technology, which involves the use of PEGylated protein mediator, may offer some advantages, such as its modular approach, it still involves covalent modification of the mediating protein and the scope of interacting proteins suitable for this purpose can be limited.

### 3.4 | Complementary pairs of PEGylated macroions

Co-assembly of proteins with two oppositely charged PEGylated polyions (Figure 4b) can potentially offer the advantage of high-environmental stability of polyelectrolyte complexes and multilayer coatings (Alkekhaia et al., 2020; Kurapati et al., 2019). This pathway typically includes protein complexation with the first polyion, similarly to methods described in previous chapters, which is then followed by adding a second polyelectrolyte of the opposite charge. The ternary polymer(A)–protein–polymer(B) assembly may be also formed using a pair of interacting nonionic macromolecules.

#### 3.4.1 | Systems on the basis of polyelectrolyte complexes

Electrostatically driven co-assembly of block copolymer with strong cationic moieties, poly(2-methyl vinyl pyridinium)-*block*-poly(ethylene oxide), weak anionic homopolymer—poly(acrylic acid), and negatively charged lipase was explored for the preparation of complexes with improved physiological stability (Lindhoud et al., 2009). Although polyion complexes themselves were stable at near physiological conditions, protein-containing nano-scale assemblies (40 nm diameter) displayed salt triggered release of lipase at 0.12 M sodium chloride. The results also indicated the adverse effect of salt on enzymatic activity of lipase in the complexes (Lindhoud et al., 2010). Inclusion of organophosphate hydrolase and  $\alpha$ -chymotrypsinogen in polyelectrolyte complexes of poly(acrylic acid) and poly(oligo(ethylene glycol) methacrylate)-*b*-poly(4-vinyl *N*-methylpyridyl iodide) was also performed to stabilize enzymes in polar organic media (Mills et al., 2016). Although the enzymes did not assemble with either polymer directly, they partitioned into the core of nanoscale micellular complex and showed improved thermal and solvent stability.

Vesicles with polyelectrolyte membrane walls, which were formed by two oppositely charged block copolymers: anionic poly(ethylene glycol)-*b*-poly( $\alpha,\beta$ -aspartic acid) (PEG-P(Asp)) and cationic poly(ethylene glycol)-*b*-poly((5-aminopentyl)- $\alpha,\beta$ -aspartamide) (PEG-P(Asp-AP)), were used to encapsulate myoglobin (Mb; Figure 7d; Kishimura et al., 2007). The resulting self-assembled microcontainers (500 nm to 5  $\mu$ m in diameter) with cavity-encapsulated protein displayed reversible oxygenation–deoxygenation of the Mb and effectively protected it against proteolytic degradation by trypsin. The two-polyion approach proved to be efficient in modulating the saline and thermal stabilities of noncovalently linked assemblies, which was achieved by tuning the hydrophobicity of aliphatic side chains in interacting macroions (Chuanioi et al., 2014). Modification of the ionic segment in a block copolymer with an octyl spacer in the side group led to a remarkable stability of complexes in the presence of 150 mM sodium chloride and at elevated temperatures. In contrast, polymers without C8 chains underwent aggregation under the same conditions. Furthermore, hydrophobically modified complexes were significantly smaller in size (60-nm diameter), when compared with their hydrophilic analogs (Chuanioi et al., 2014).



As evident from above, copolymers containing poly(amino acid) domains remain some of the most popular choices for use in both binary and ternary PEGylated systems, which is dictated by the ability of those macromolecules to undergo biodegradation. However, such degradation is generally limited to microbial and enzymatic mechanisms (Kimura & Fujimoto, 2010; Obst & Steinbüchel, 2004). Polyacids are generally resistant to conventional proteases or peptidases with at least some of them showing lack of significant degradation either in vitro, with isolated enzymes and homogenates, or in vivo (Kimura & Fujimoto, 2010; Rypáček et al., 1997). Furthermore, enzymatic degradation can be potentially challenging to control and can lead to irreproducible in vivo performance (Staubli et al., 1990). To that end, noncovalent complexes designed on the basis of hydrolytically degradable polyions can streamline the pathway to clinical development.

With this objective, a series of PEGylated polyphosphazenes containing either cationic dimethylaminopropoxy (CP-PEG) or anionic carboxylatoethylphenoxy side groups (AP-PEG) have been synthesized and explored for their ability to noncovalently modify an important therapeutic enzyme—L-asparaginase (Figure 8a; Andrianov et al., 2018). Since the isoelectric point of this enzyme is relatively low (4.6–5.5; Narta et al., 2007), L-asparaginase was first treated with cationic CP-PEG and then additionally modified with anionic AP-PEG to achieve electroneutrality of nano-sized complexes and avoid potential toxicity of the positively charged species. The resulting ternary complexes showed no sign of aggregation and preserved enzymatic activity of L-asparaginase. In vitro testing of the noncovalently PEGylated enzyme demonstrated desirable suppression of the antigenicity, with the degree of its reduction inversely related to the content of its cationic component. Complexes also provided for the protein protection against proteolytic degradation, which also benefited from the addition of a second polyelectrolyte (Andrianov et al., 2018). Furthermore, in vitro half-life of noncovalently PEGylated L-asparaginase in formulations containing trypsin exceeded that of the native enzyme over 8.5-fold, which was consistent with the covalent PEGylation effect that typically increases L-asparaginase stability ~7- to 10-fold (Narta et al., 2007). Both anionic and cationic polyphosphazenes displayed temperature dependent degradation profiles, which provided for 2 week to one-and-a-half months half-life (dependent on the polymer) at near physiological conditions (pH 7.4, PBS, 37°C), as well as adequate shelf-life under refrigeration (Andrianov et al., 2018).

### 3.4.2 | Systems utilizing nonionic interactions

A two-stage noncovalent method for PEGylation of insulin was described, in which this peptide hormone was co-assembled into a three-component complex (Li et al., 2018). First, zinc-chelated poly(aspartic acid-co-aspartylglucosamine-co-aspartyltrinitrotri-acetic acid) was complexed with insulin via histidine groups of the peptide. Next, PEG containing poly(ethylene glycol)-*b*-poly(aspartic acid-co-aspartylamidophenylboronic acid) block copolymer was added, which resulted in micelles of submicron dimensions (122–187 nm in diameter) stabilized with a PEG shell. An insulin-containing core of micelles was formed via cross-linking of phenylborates of PEGylated polymer and cis-diols on glucosamine. The glucose triggered pulse release of insulin was achieved due to the ability of phenylboronic acid moieties to bind glucose and undergo a transformation from a neutral hydrophobic complex to a negatively charged hydrophilic state. In vivo studies of PEGylated complexes in type 1 diabetes, streptozotocin-induced diabetic mice, demonstrated ability of noncovalently PEGylated insulin to regulate blood glucose levels. The blood glucose levels in animals treated with PEGylated insulin was maintained in a normoglycemic state (<2.0 g/L) for about 5 h, which was much longer than that of free hormone—about 1 h (Li et al., 2018).

This approach was further extended by replacing glycopolymer fragments with poly( $\epsilon$ -caprolactone) segments (Li et al., 2020). The hydrophobicity of caprolactone chains induced spontaneous micelle formation at physiological pH. When such self-assembled micelles with confined hydrophobic domains on the surface were formulated with insulin, the peptide adsorbed on the confined hydrophobic domains of micelles, which prevented its aggregation and fibrillation. This process resembled binding and stabilization of proteins by natural chaperons. Insulin-loaded synthetic nanochaperones showed an effective therapeutic efficacy on diabetic mice (Li et al., 2020).

### 3.5 | PEGylated dendritic copolymers

Another promising example of the PEGylation system, which is entirely based on noncovalent interactions, included the use of dendritic copolymers (Wang et al., 2016). A series of 11 copolymers—telodendrimers (Figure 8b)—were synthesized, which integrated various combinations of charged functionalities: cationic—arginine and lysine, anionic—

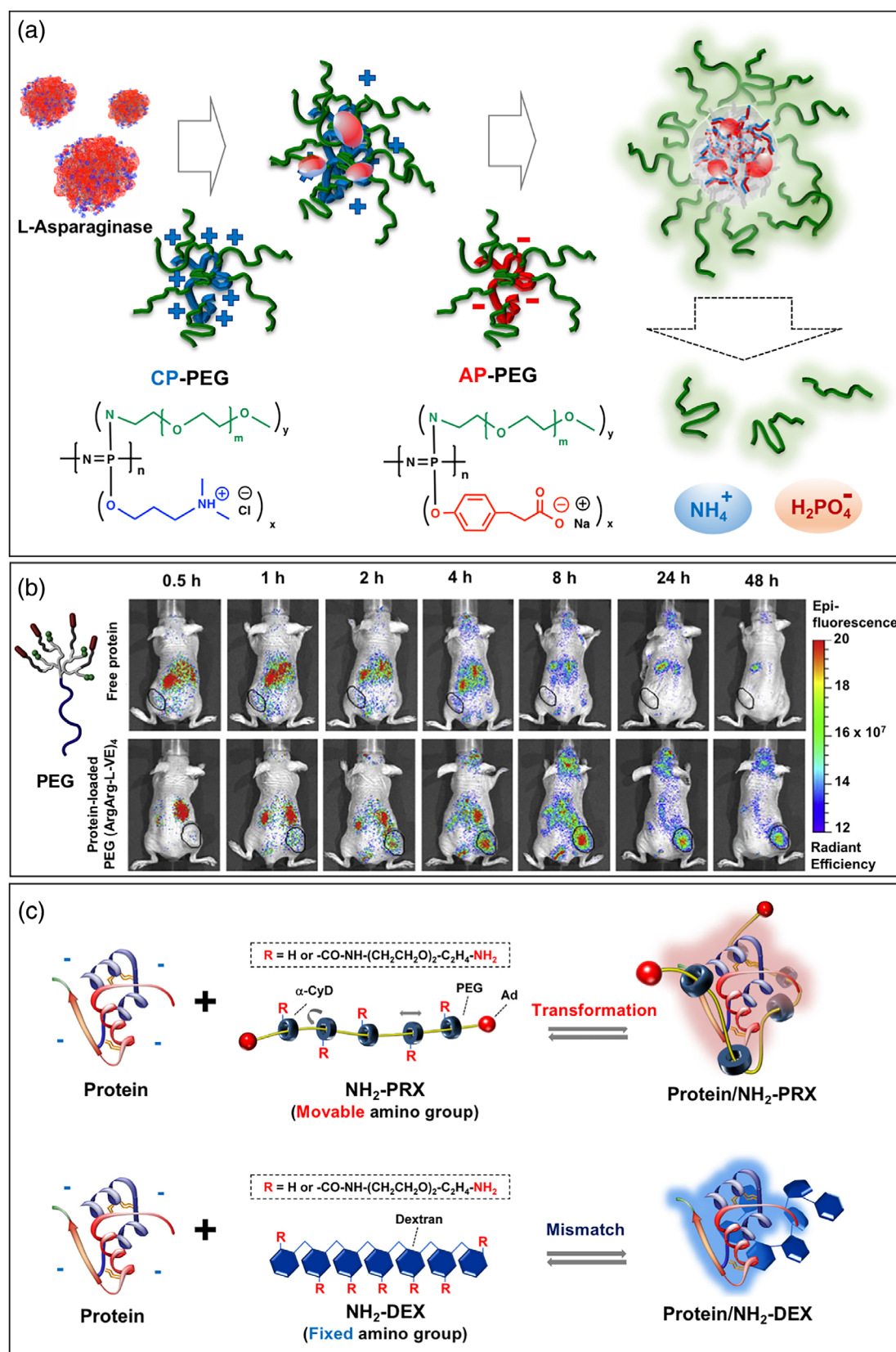


FIGURE 8 Legend on next page.

oxalic acid, and hydrophobic functionalities, such as heptadecanoic acid, cholesterol, and tocopherol. Their interactions with proteins were studied using electrophoresis, isothermal titration calorimetry, Forster resonance energy transfer spectroscopy, bio-layer interferometry, and computational methods. The resulting protein-loaded nanoparticles, with dimensions of <30 nm, displayed efficient cell-penetrating capability. Furthermore, the noncovalently bound nano-assemblies efficiently delivered proteins to xenografted tumors by the “enhanced permeability and retention” (EPR) effect and improved retention of peptides in an orthotopic brain tumor. In vivo animal imaging of mice bearing xenograft HT-29 colon cancer indicated that intravenously administered noncovalently modified proteins efficiently targeted tumor (Figure 8b). In contrast, mice, which received injections of unmodified insulin showed fast protein clearance and low tumor uptake. Authors noted the importance of synergistic effects of multivalent ionic and hydrophobic functionalities of telodendrimers in enhancing protein-binding affinity and the role of hydrophobic groups as annealing moieties in stabilizing protein binding in aqueous solutions (Wang et al., 2016). The telodendrimers approach has been further advanced to the modification of polycationic vector technique for intracellular delivery (Wang et al., 2018). The model protein BSA was co-assembled with branched polyethylene imine (BPEI) and the resulting complex was further modified with PEG-containing telodendrimer. Sheltering positive charges by PEG resulted in the reduction of cytotoxicity and hemolytic activity, but nevertheless allowed for efficient intracellular protein delivery (Wang et al., 2018).

### 3.6 | PEGylated copolymers with mobile side groups

The critical importance of side group mobility for the efficient polyelectrolyte–protein complexation was demonstrated using a PEGylated macromolecule, in which cationic moieties and PEG grafts were attached to the PEG backbone not covalently, but via cyclodextrin (CyD) macrocycles threaded through the backbone—polyrotaxane structural design (PEG-PRX) (Utatsu et al., 2021). Interactions of PEG-PRX with proteins were benchmarked against similarly functionalized polymer with a rigid dextran backbone (PEG-DEX; Figure 8c). Although both polymers formed noncovalent complexes with proteins, such as insulin, panitumumab, and lysozyme, the efficiency of protein uptake was significantly higher for PEG-PRX. In vivo hypoglycemic effects of noncovalently PEGylated insulin were studied in healthy and GK/Slc type 2 diabetic rats. Both polymers prolonged low serum glucose level compared with unmodified protein in either animal model, but, once again, the effect of PEG-PRX was more pronounced illustrating the importance of side groups mobility (Utatsu et al., 2021). Authors further note that with the graft copolymer design used in the study, the molecular mass of 2 kDa PEG may be sufficient for avoiding glomerular filtration. Safety studies undertaken in rats following four intravenous administrations demonstrated that PEG-PRX did not induce severe adverse effects (Kogo et al., 2022).

## 4 | CONCLUSION

The noncovalent PEGylation approach has the potential to offer several advantages over traditional covalent methodology. The successful noncovalent technology can drastically reduce the cost of PEGylated drugs and improve their pharmacokinetic profiles, which appears to be possible due to the dynamic nature of protein modification. The simplicity of the formulation approach, which can potentially lead to the development of “mix-and-match” therapeutics, may further advance the emerging field of personalized medicine and extend the scope of protein therapies that are currently considered to be difficult to PEGylate covalently. It can also be envisioned that the transient association of PEG with the protein can simplify regulatory approval. The achievement of adequate protein–PEG association and potential instability of noncovalently PEGylated drug under physiological conditions may not negatively impact applications relevant to

**FIGURE 8** (a) Schematic presentation of noncovalent PEGylation of L-asparaginase with a complimentary pair of polyphosphazene polyions and their degradation. Adapted with permission from Andrianov et al. (2018). Copyright © 2018. American Chemical Society. (b) Systemic delivery of telodendrimer-modified insulin. In vivo animal images of the HT-29 colon cancer-bearing nude mice xenograft models (#A1 and #A2) after tail vein injection of free cyanine labeled (Cy5) insulin, and Cy5-insulin-loaded telodendrimer nanoparticles. The black circles in (a) indicate the tumor sites. Adapted with permission from Wang et al. (2016). Copyright © 2016. Elsevier Ltd. (c) Preparation of protein–polyrotaxane (PEG/NH<sub>2</sub>-PRX) and protein–dextran (PEG/NH<sub>2</sub>-DEX). Reprinted with permission from Kogo et al. (2022). Copyright © 2022. American Pharmacists Association. Published by Elsevier Inc.

improving protein shelf-life but continue to present serious challenges for the clinical development of the technology. To that end, the advantage of high-affinity binding, such as host–guest complexation, and multivalent-based approaches in particular, appear to hold a great promise. Since the latter mainly rely on the use of polymers, especially polyelectrolytes, there is a clinically dictated need to introduce water-soluble macromolecular systems with hydrolytically degradable backbones. Hydrolytic degradation of protein interactive PEG systems can also reduce concerns associated with potential reactogenicity of modified PEGs and increase the possibility of their approval for clinical use. Furthermore, the mobility of interacting side groups and protein–PEG linkage may play a significant role in stabilization of the complex. This can be achieved either with mechanically interlocked molecular architectures, as was elegantly demonstrated using polyrotaxane design, or polymers with highly flexible backbones, such as phosphazene- or siloxane-based macromolecules. Recent studies also suggested that in the design of the appropriate protein–PEG complexes, the route of administration should be carefully considered. It has been demonstrated that the same supramolecular formulation may produce drastically different therapeutic effects when administered subcutaneously or intravenously.

The interplay between stealth characteristics of the PEGylated protein and its biological activity, which presents a serious challenge and impediment for covalent PEGylation due to their inverse relationship, is not sufficiently studied for noncovalently assembled systems. It is highly encouraging that recently emerged data suggest highly dynamic nature and reversibility of interactions between the protein and its noncovalently attached PEG shield, which result from host–guest and polymer–polymer exchange reactions. Further research in this field is needed, especially studies focusing on the mechanism and kinetics of exchange reactions and general stability of noncovalent complexes in the presence of plasma and its main constituents. Moreover, the efficiency of the approach needs to be compared with that of the releasable covalent PEGylation technology, which provides for the regeneration of authentic and fully active protein drug over time (Filpula & Zhao, 2008; Gong et al., 2015). The validity of noncovalent PEGylation approach should be especially carefully considered when applied to heterologous proteins, which may trigger dramatic immunogenic responses or even anaphylactic reactions. Finally, the ongoing concern on clinical safety of PEG, which appeared to intensify due to recent studies on anti-PEG antibodies (Hong et al., 2020; Huckaby et al., 2020; Kozma et al., 2020), cannot be neglected. In fact, the antibody-mediated immune reactions to PEGylated macromolecules range from local inflammation to severe reactions, such as complement-mediated immediate type hypersensitivity with occasionally fatal anaphylaxis (Kozma et al., 2020; Shi et al., 2022). Protein-binding properties of modified PEGs can potentially exuberate this issue. This dictates the need for studying a noncovalent approach with other promising stealth alternatives (Hoang Thi et al., 2020), such as polyoxazolines (Bludau et al., 2017; de la Rosa, 2014) and zwitterionic systems (Blackman et al., 2019; Qu et al., 2022). In fact, a recent publication already describes the attachment of zwitterionic polypeptide structures based on the naturally occurring amino acid methionine to the protein via cucurbit[7]uril conjugation (Clauss et al., 2023). Overall, given the rapid emergence of versatile noncovalent methodologies and their evolving roles in the field of drug delivery and biomaterials (Webber, Appel, Meijer, & Langer, 2016; Webber & Langer, 2017), it is reasonable to expect significant advances in the development of noncovalently enabled stealth technologies in the coming years.

## AUTHOR CONTRIBUTIONS

**Alexander Andrianov:** Conceptualization (lead); formal analysis (lead); writing – original draft (lead); writing – review and editing (lead).

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## CONFLICT OF INTEREST STATEMENT

The author has declared no conflicts of interest for this article.

## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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