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Protein-polymer therapeutics: a macromolecular perspective

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Protein-Polymer Therapeutics – A Macromolecular Perspective

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The development of protein-polymer hybrids has, several decades ago, emerged with the vision that their synergistic combination will offer macromolecular hybrids with manifold features to succeed as the next generation therapeutics. From the first generation of protein-polymer therapeutics represented by PEGylated proteins, the field has since advanced, reinforced by the progress in contemporary chemical techniques for designing polymeric scaffolds and protein engineering. Novel polymerization techniques that offer multifunctional strategies as well as a greater understanding of proteins and their biological behavior have both proven to be exceptional tools in the construction of these hybrid materials. In this review, we seek to summarize and highlight the recent progress for these semi-synthetic protein hybrids in terms of their preparation, design, resultant bioarchitectures and bioactivities for their intended bio-applications.

1. Introduction

Biopharmaceuticals such as proteins, nucleic acids and lipids are eminent candidates in the development of macromolecular drugs for the treatment of various diseases. They generally offer the advantage of biocompatibility and biodegradability since many of them are naturally produced by the human body and hence well tolerated and excreted.[1] Proteins have found a representation among the majority of the FDA approved biopharmaceuticals since the historic introduction of insulin for the treatment of diabetes mellitus type I/II and the advancement in recombinant technology.[2] In contrast to small molecular drugs, protein-based treatment is exceptionally specific and their biological behaviour is predictable, resulting in a shorter time required for FDA approval.[1]

Although proteins have emerged as target-specific therapeutics, most of them still lack proteolytic stability and sufficiently long circulation times.[3] Moreover, the efficacy of antibody-based treatment can be adversely affected should the patient possess a compromised immune system due to chemotherapy and/or other health complications (e.g. HIV, auto-immune diseases).[4] In order to further enhance the pharmacological behaviour and stability of protein therapeutics, protein-polymer conjugates have since been investigated as contemporary biohybrid materials with the aim of achieving synergistic effects between the natural and synthetic components.[5] Ideally, the vast combinations of polymeric architectures, their lengths, branching, side chain functionalities and stimulus responsiveness provide a multi-dimensional platform for chemical design while proteins complement with unrivalled target specificity and bioactivity. As a consequence, the engineering capacity provided by synthetic polymers can be used to alleviate the common physical limitations (temperature, pH and degradation) of proteins and in many recent cases, modulate their trafficking and activity to tailor each individual need.[5] The inception of polymer-protein hybrids for cancer treatment was displayed by the seminal study by Matsumara and Maeda on the effect of polymer conjugated neocarzinostatin (SMANCS) in tumor tissues establishing the first experiments on the phenomena that is now widely known as the enhanced permeability and retention effect.[6] Several tumor physiological studies promptly followed this discovery to investigate the mechanism of tumor vasculatures and have identified physical structural differences that promote accumulation of nanometer size molecules or particles thus propelling the development of polymer-protein conjugates.[7, 8] Apart from using polymers purely as high molecular weight substituents that can greatly enhance pharmacokinetic parameters, their role as functional entities imparting completely novel features has emerged over the past years.[9] Hence, with the availability of many different polymers or copolymers of varying monomer sequences and topologies, there is a considerable and still mainly undiscovered chemical space that offers access to biomacromolecular hybrid architectures with manifold features and bioactivities.[5]

In this review, synthetic concepts for achieving protein-polymer conjugates are introduced that allow the preparation of topologically diverse protein hybrid architectures applying both covalent and non-covalent conjugation reactions. The opportunity to vary and tailor protein-polymer architectures represents an eminent strategy to impart unique features and greatly enhance the spectrum nature offers. The relatively young branch of chemical protein engineering is still emerging and provides great opportunities to adapt concepts of nature into macromolecular constructs that display attractive and often unexpected biological activities. Applications of such semi-synthetic protein hybrids as macromolecular therapeutics are obvious and main strategies will be discussed in this review article.

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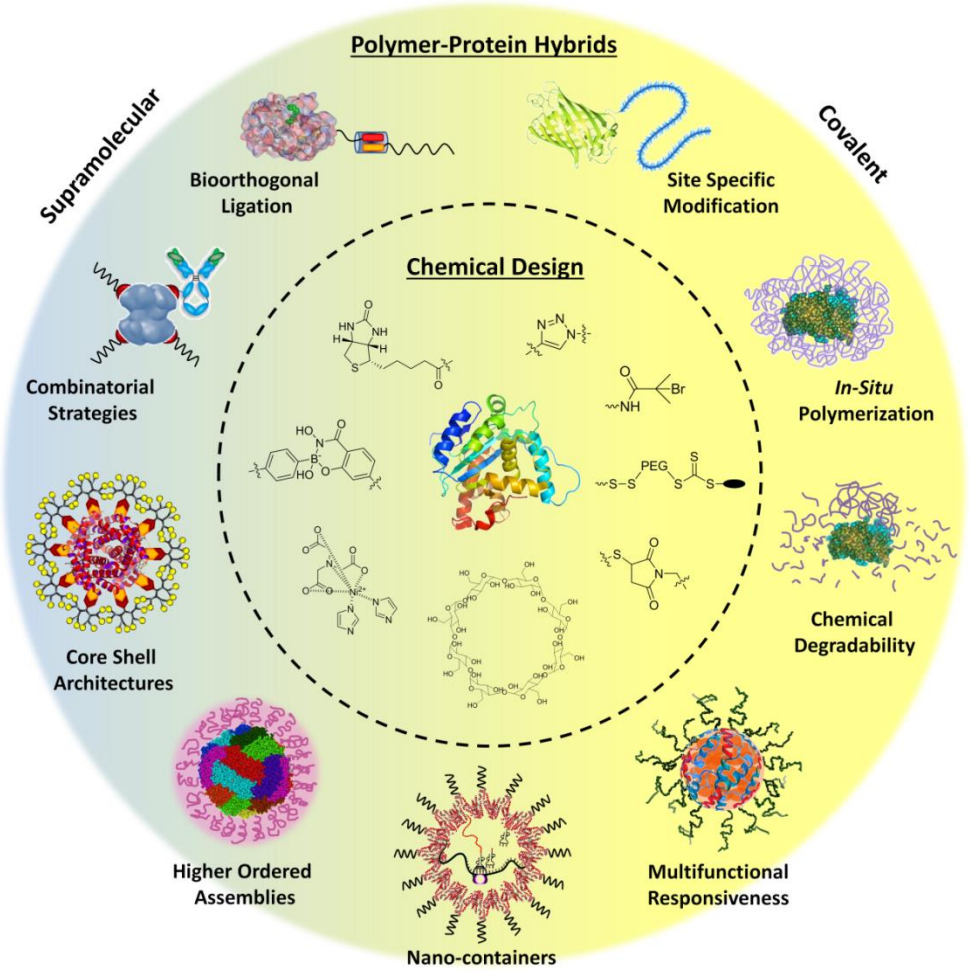

Figure 1. Overview of different protein-polymer architectures prepared by supramolecular chemistry and covalent conjugations.

2. Protein-Polymer Conjugates – Synthesis Concepts and Dispersity

2.1 Preparation of Covalent and Supramolecular Protein-Polymer Conjugates

The conjugation of polymers and proteins proceeds either via covalent reaction and/or non-covalent supramolecular chemistry, each presenting its advantage in addressing certain biological events with the vision of creating personalized medicine (Figure 1). For covalent modification, the protein surface offers a pool of functionalities (amines, carboxylic acids, alcohols, thiols etc.) facilitating both statistical and site specific chemical techniques. Should a statistical modification of the protein surface be desired, functionalization is usually accomplished targeting at the amine or carboxylic acid residues of lysines, glutamate or aspartate side chains, respectively.[10] In the case where precise surface chemistry is essential, accessible cysteine residues, disulfide bridges, N-terminal lysines, less abundant tyrosine or unnatural amino acids can be addressed and modified.[10] Primarily, covalent conjugates are mostly stable under physiological conditions, with notable exceptions such as esters and disulfide bonds, which can be degraded by esterases and glutathione, respectively, within the body.[11, 12] Nonetheless, the selection of chemistry for the attachment of polymeric macromolecules without compromising protein structure and activity remains a primary concern.[13, 14]

In this aspect, reversible modification of proteins with polymers via supramolecular interactions provides the possibility of releasing polymers from the protein core under designated conditions.[15] Consequently, the modified protein could potentially benefit from improved stability due to the polymer modification and regain their full bioactivity upon release.[16] In addition, non-covalent interactions also allow the assembly of

proteins into higher ordered architectures providing defined activities that are unique from their molecular constituents.[17] As a result, supramolecular chemistry involving both chemical based interactions (e.g. hydrogen bonding, electrostatic interaction, metal chelation etc.) and those of biological origin (e.g. protein-substrate/inhibitor interaction, protein-cofactor interaction etc.) have received increasing attention for the development of non-covalent ligation strategies and triggered assemblies, e.g. ligand-directed labeling strategies.[18, 19] Through these affinity based conjugation methods, unspecific chemical reactions at the surface of proteins can be minimized or, in some cases completely eliminated by utilizing specialized binding proteins e.g. streptavidin.[20]

In consideration of both covalent and non-covalent techniques, three major strategies - *grafting to*, *grafting from* and *grafting through*, are implemented (Figure 2).[21] Direct attachment of synthetic polymers to the target protein, so called *grafting to* strategy, represents the most common and straightforward methodology.[22] Poly(ethylene)glycol (PEG)-based conjugates are exclusively prepared by the *grafting to* strategy using covalent protein labeling chemistry (e.g. thiol-maleimide, succinimidal esters),[23] which will be discussed in details in section 3.1. Due to its benign pharmacological properties (non-toxic, non-immunogenic), a large hydrodynamic contribution and enhanced pharmacokinetics, PEGylated proteins are the first examples of hybrid conjugates that have been widely explored.[24] The prime advantage of the *grafting to* approach is that the polymer component can be synthesized separately in a non-aqueous environment prior to the final conjugation step. As a consequence, it offers the most diverse range of chemical functionalities and combination of monomers for all polymerization strategies.

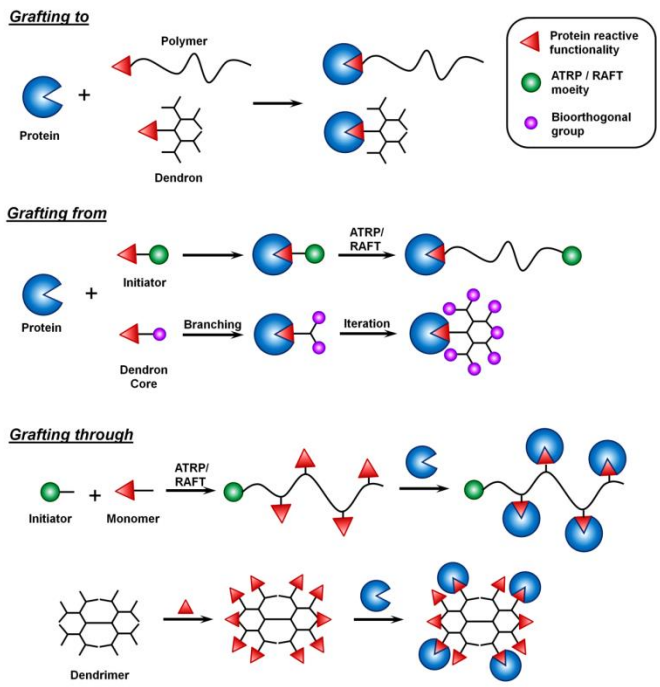

Figure 2. Three main synthetic approaches for the construction of protein-polymer hybrids.[25]

Independently, the development of the *grafting from* and *grafting through* approaches have been propelled recently particularly due to major advances in "living polymerization" techniques focusing on controlled polymerization such as atom transfer radical polymerization (ATRP) and reversible-addition fragmentation transfer (RAFT).[25] For the *grafting from* strategy, the polymerization initiator is firstly conjugated onto a protein and living polymerization is then induced from this initiator to afford controlled growth of the polymer chain from the protein core. Although polymerization reactions in the presence of protein requires highly challenging biocompatible aqueous conditions, the unique advantages including the possibility of preparing a narrowly dispersed polymer fitting to the protein scaffold and the ease of purification, makes this strategy highly attractive to biomaterial scientists.[5] On the other hand, as opposed to conjugating several polymers onto one protein core, the *grafting through* approach allows the connection of several proteins onto one polymer chain taking advantage of protein multivalency. Protein interacting entities (e.g. protein reactive functional groups, affinity tags such as biotin) are firstly copolymerized into one polymer segment allowing the proteins to be subsequently attached along the polymer main chain to form multivalent proteins.[26]

2.2 Dendrimer Protein Conjugates – A Monodispersed Alternative

In addition, it is apparent that the synthesis of protein-polymer hybrids is not restricted to linear polymers. Regularly branched polymers, dendrimers, have emerged as an attractive class of macromolecules as they, unlike conventional polymers, are monodispersed and possess a defined molecular architecture.[27] As a result, the elucidation of their respective biological characteristics is less complex and the construct's response to changes in its environment as well as potential supramolecular interactions with other biomolecules found within the human body can be better predicted.[28] From the physical perspective, dendrimers possess a defined correlation between their molecular dimension and generation number, increasing by 1.2 nm with each generation for the polyamidoamine (PAMAM) scaffold, which represents one of the best studied dendrimers.[29] Hence, the design of a dendrimer-protein hybrid is significantly more precise when customizing size and surface functionalities, which are determining factors in biomedical applications.

The strategies for the creation of dendrimer-protein constructs, in principle, directly follow those of protein-polymer hybrids albeit with a variation in the type of chemistry involved. In comparison to linear polymers, the tradeoff for constructing monodispersed dendrimer-protein conjugates is that the step-wise synthesis and purification of these perfectly branched macromolecules is significantly more challenging.[28] Despite this compromise, dendrimer based biohybrid materials have been growing steadily, ranging from peptide and protein bioconjugates[30] to dendronized nucleic acids[31] hybrid architectures. Synergistic features and applications as precision therapeutics will be discussed in greater detail in section 3.4.

Together, these versatile synthetic approaches have led to the construction of extraordinarily diverse protein-polymer architectures and have proven invaluable in all areas of therapeutic applications. The next two sections will discuss the representative examples of each type of protein-polymer architectures highlighting their preparation methods and application potential.

3. Covalent Protein-Polymer Conjugates: Complementary Properties and Biomedical Evaluation

3.1 Single Chain Protein Polymer Hybrids for Protein Delivery

Site-specific conjugation of only a single polymer chain remotely away from the protein active centre minimizes its influence on protein activity and is often the preferred strategy compared to statistical modification (Figure 3a).[32, 33] In clinical practice, the conjugation of a single high molecular weight polymer chain has proved adequate to adjust the pharmacokinetic properties of therapeutic proteins and instil macromolecular benefits.[34, 35] For instance, due to the highly hydrated nature of PEG polymers, it is reported that the conjugation of a single PEG chain could efficiently increase the hydrodynamic radius of the protein conjugate, which could be approximately 5–10-fold larger than it would be predicted from the molecular weight alone.[36, 37] Site-specific PEGylation of therapeutic proteins was mainly achieved by covalent conjugation via *grafting to* strategy, which relies on targeting chemoselective anchor groups e.g. cysteines,[38] disulfides[39] or the N-terminal amine group. Since free cysteines are not often present in native proteins, introducing free cysteine residues through point mutation has been a common approach to accomplish site-specific protein modification in the last decade.[40] Several therapeutic proteins, recombinant immunotoxins[41] and Cyanoviri-N, a potent inhibitor of human immunodeficiency virus (HIV),[42] have been site-specifically PEGylated via this approach and the resultant constructs demonstrated increased activity *in vitro* and *in vivo*. Another site-specific PEGylation method targets the N-terminal amine group, which displays a lower pKa value (pKa 7~8) compared to the side-chain counterpart (pKa 9~10).[34, 35] However, the selectivity between the N-terminal amine and lysine side-chains is not optimal in many proteins, therefore genetic engineering to decrease the number of lysine residues is often required to achieve highly site-specific bioconjugation.[43] In this aspect, Brocchini et al. have developed a chemical method to specifically modify and rebridge disulfide linkages in proteins through a bis-sulfone linker.[39, 44] Site-specific PEGylation of human interferon α2b (IFN-α2b) and a fragment of an antibody to CD4+ was reported with this method both showing the retention of tertiary structure and biological activity of these proteins.[39] This disulfide modification has been found reversible and can be replaced in presence of high glutathione concentration as present in many tumors, which would in principle allow releasing the polymer chain in cancer cells and regenerating the native protein after delivery.[45] A convenient bifunctional linker based on this chemistry was recently reported that allows step-wise crosslinking the disulfide bridge of the protein and the thiol-group of the polymer chain by simply switching the pH thus further broadening the application of disulfide intercalation.[46] However, it is important to note that should the protein active site be close to the targeted disulfide, conjugation of polymers via this method will cause a significant reduction of protein activity, with the PEGylated IFN-α2b displaying a loss of 90% activity compared to the native protein.[39] Some other site-specific PEGylation strategies including tag-based selectivity[47] and enzymatic reactions,[48] have also resulted in a few successful examples of mono-PEGylated proteins, such as PEG-interferon[49] and PEG-transglutaminase.[48] Nevertheless, it should be noted that research in this direction mainly focuses on methodology development. While *in vitro* activities of the conjugated proteins have been investigated, the *in vivo* effects after site-directed conjugation still require deeper evaluation. Furthermore, since the currently available site-specific chemical methods are still confined to proteins with specific structural features, such as limited number of chemo-accessible reactive groups, and are not generic methods for proteins, substantial efforts are required to develop more robust strategies to meet complex clinical requirements.

Over several decades, PEG has been almost the only polymer option to enhance pharmacokinetics of therapeutic proteins without detrimental side effects such as toxicity and biocompatibility. However, PEGs acquire certain limitations as they are mainly prepared by ring opening polymerization with high polydispersity,[50] they are only to be conjugated onto proteins via the *grafting to* strategy and they are not biodegradable.[23] Functionally saturated, they offer no further design possibilities to integrate additional physical or chemical properties.[51] In recent years, the diversity in polymer scaffolds designed for the construction of hybrid materials have risen sharply owing to the advancement of polymer preparation methods and the investigation of other unique polymer scaffolds rivalling that of PEG. Several examples of PEG like polymers reported overcoming the limitations of conventional PEGs. For instance, polyglycidols contain a PEG like backbone and functional side chains have been studied as functional PEG analogues[52] or copolymerized with PEG to introduce functional side chains to PEG block copolymers.[53, 54] In order to allow PEGylation of protein by the *grafting from* strategy, polymethacrylates with PEG like residues, e.g. poly(oligoethylene glycol) methyl ether methacrylate), were reported allowing *in situ* polymerization from the protein core via atom transfer radical polymerization (ATRP).[55] Another interesting class of PEG-like polymers, which, however, so far has been barely explored for the synthesis of protein − polymer conjugates, are polyglycerols (PGs).[56] PGs can be prepared with linear or hyperbranched structures thus providing access to libraries of chemically identical but structurally different polymers for optimizing the pharmacokinetic properties and improving therapeutic effects.[56]

In the pursuit for PEG alternatives, oligosaccharides, inspired by the glycosylation of proteins present in nature, have been also considered as PEG analogues.[57, 58] Likewise, oligosaccharides provide a large hydration shell capable of protecting proteins from proteolysis and introducing prolonged circulation times.[59] However, the tedious synthesis of carbohydrate derivatives that can be used in protein glycosylation and the poor solubility of large molecular weight carbohydrates swiftly became the bottleneck in the development of glycosylated therapeutic proteins.[60] Therefore, only few synthetic oligosaccharide-protein conjugates have been studied. For instance, sialylactose was site-directly conjugated to point mutated insulin without loss of biological activity[57] and *in vivo* tests with mice clearly demonstrated the prolonged glucose-lowering activity in the blood stream. A facile method has been proposed by Wang et. al. to site-selectively glycosylate hemoglobin at a free cysteine-93 position with large molecular weight glycans.[61] This method allows rapid functionalization of carbohydrates with thiol reactive maleimides via click reaction with cysteine containing proteins, and was proposed as a potential alternative to PEGylation.[58] Enzymatic reactions could also be used for site-specific glycosylation. Bertozzi et. al.[62] demonstrated a site-specific antibody glycosylation by genetic introducing a peptide tag that could be recognized by formylglycine generating enzyme to convert a cysteine to aldehyde-bearing residue formylglycine (fGly) during coexpression in E. coli. This engineered aldehyde group thus allowed further conjugation with aminooxy N-acetylglucosamine and further oligosaccharide elongation with an engineered glycosynthase.[62] These sequential enzymatic reactions would be especially attractive when highly

specific and efficient glycosylation is required on E. coli expressed proteins. Recently, recombinant approaches to achieve glycopolymer-based therapeutic proteins have even been marketed.[63] The DNA sequence encoding a biologically active peptide or protein is ligated to a DNA sequence encoding a specific glycopolymer polypeptide (e.g., repeating amino acid sequences of Asp-Asp-Thr (NNT) or Asp-Asp-Ser (NNS)). The final ligated gene sequence is cloned into mammalian recombinant protein expression systems and the resultant glycoprotein possesses many N-linked glycosylations on the glycopolymer domain due to the presence of the repeating NNT or NNS amino acid sequences.

On the other hand, synthetic polypeptides also represent an important class of polymers that is promising for therapeutic protein delivery. Polypeptides are generally considered more biocompatible than other synthetic polymers and they are biodegradable by endogenous enzymes. One unique advantage of using polypeptides for protein modification is the application of recombinant techniques to obtain site-specific protein-polypeptide conjugates. The bioengineering method provides an attractive alternate option for preparing protein-polymer hybrid materials. For instance, cationic polypeptides, such as poly-L-lysine (PLL) and poly-L-arginine (PLA) have shown enhanced cell membrane penetration and intracellular delivery of the active proteins, e.g. human catalase, and when the fusion protein was sprayed on animal skin, increased penetration through the epidermis and the dermis of the subcutaneous layer was observed.[64] However, polycations often induce various toxicities such as mitochondria-mediated apoptosis,[65] which might limit (pre-) clinical development. Recently, the Chilkoti group has investigated elastin-like polypeptides (ELPs) as a class of stimulus-responsive polypeptide that many attractive features for *in vivo* drug delivery and tissue engineering applications. They have reported optimized protocols for the attachment of ELPs to proteins by fusion of a gene encoding an ELP with that of the protein of interest,[66] and found that the ELP improves the systemic pharmacokinetics and biodistribution of the protein, or it can be used as an injectable depot for sustained, local protein delivery.[66] Cationic amino acids have also been included in ELP tags to optimize the cellular uptake of the conjugated protein.[67] Such supercharged ELPs offer reduced toxicity *in vitro* compared to conventional polycationic peptides such as PLL and PLA due to reduced charge densities along the peptide chain.[67] Likewise, proline/alanine-rich sequences (PAS) have been coupled to proteins and peptides by chemical or recombinant approaches.[68] They adopt a stable random-coil structure with expanded hydrodynamic volume under physiological buffer conditions at ambient or body temperature. PASylation of proteins has been commercialized[69] recently due to extended plasma half-lifes of the resulting conjugates similar as PEGylation.

A few examples of other polymers have also been investigated. For instance, the anticancer protein neocarcinostatin conjugated with a copolymer of styrene has been approved as a protein drug for the treatment of hepatocellular cancer in Japan.[70] More recently, a zwitterionic polymer, poly(carboxybetaine), has been reported for protein modification that surpasses PEG in terms of anti-fouling capabilities, protein stabilization while simultaneously maintaining protein activity and binding affinity.[71] The further development of these alternative polymers options will open substantial opportunities to broaden the applications of protein-polymer therapeutics.

3.2 Protein-Polymer Hybrids for Stimuli Responsive Enzyme Switches

In addition to polymers conjugation at a location opposite to the active site, the conjugation of polymers nearby the ligand binding site offers adjusting protein functions on demand (Figure 3b). Stimuli responsive polymers responding to light, temperature and/or pH have been incorporated as activity regulators offering unique opportunities to alter protein characteristics.[72] Several interesting biohybrid architectures have been developed by Hoffman, Stayton and co-workers:[73,74] A cysteine mutation has been introduced at the active site of endoglucanase, which was conjugated with a photoresponsive ((*N*,*N*´-dimethylacrylamide)-co-4-phenylazophenyl acrylate) polymer affording a photoswitchable protein hybrid.[73] The resultant modified endoglucanase catalyzed glycoside hydrolysis when irradiated with UV light at 350 nm but it turned inactive under 420 nm depending on the conformation of the conjugated azobenzene moiety (Figure 3b).[73] Similarly, the thermal responsive polymer poly(*N*-isopropylacrylamide) (pNIPAm) was conjugated to streptavidin at a mutated cysteine residue close to the biotin recognition site.[74] The high affinity of the biotin and streptavidin interaction was maintained below 32 °C, but no interaction was observed above the lower critical solution temperature (LCST) of pNIPAm as the collapse of the polymer chain blocked the binding site.[74] In addition, the observed thermal sensitivity was fully reversible for several cycles.[74] Owing to their chemical orthogonality and mild reaction conditions, poly(NIPAm) was also conjugated to proteins following the *grafting from* strategy. Barner-Kowollik et al. exemplified the retention of BSA esterase activity upon *in situ* RAFT polymerization of pNIPAm.[75] The physical properties of the resultant pNIPAm-BSA hybrids displayed temperature dependent characteristics, reversibly forming larger size particles above their LCST.[75] Other thermal responsive polymers, e.g. poly(sulfobetaine methacrylamide) (pSBAm)[76] and poly[*N,N'*-dimethyl(methacryloylethyl) ammonium propane sulfonate] (pDMAPS),[77] have also been used for enzyme modification. By using two consecutive ATRP reaction from the surface of chymotrypsin, Russell et. al. could tailor the block copolymer pSBAm-block-pNIPAm to impart a double layer shell responsive to two different temperatures and control enzyme activity in a narrow window.[76] These examples demonstrated great potential of using smart polymers to create chemically engineered enzymes with adaptable properties. However, the bulk of these studies are investigated at the *in vitro* level and the elucidation of the *in vivo* biological behavior for these smart protein-polymer hybrids is currently in progress. At present, the introduction of a broader range of responsive polymers is still limited by the synthesis of stimuli responsive polymers with appropriate functional groups, the challenge to conjugate polymers to the desired location at the target protein, low solubility, undesirable aggregation, toxicity and lack of biocompatibility.

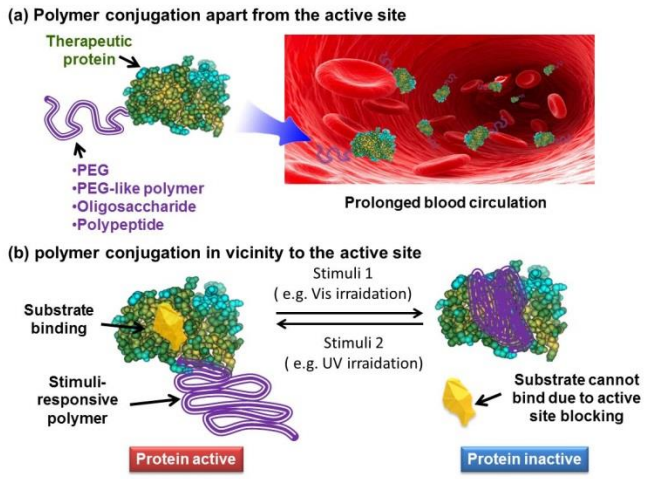

Figure 3. (a) Site-specific conjugation of a polymer apart from the active site to gain prolonged blood circulation. (b) Site-specific conjugation of a stimulus responsive polymer nearby from the active site and control of catalytic activity by the stimulus.

3.3 Protein-Polymer Core-Shell Architectures

The conjugation of multiple copies of a polymer onto the protein surface results in the formation of core-shell protein-polymer architectures. In Nature, bacterial toxins are often protected by a shell of haemaglutinin proteins that are assumed to protect the toxin from degradation at acidic pH in the stomach.[78] Inspired by the efficacy of this unique assembly, polymer shells surrounding the protein core have been introduced to achieve a macromolecular protection of the inner protein and to impart desirable pharmacokinetic properties or additional recognition functions (Figure 4a). Specifically, the polymer shell imparts desired interaction sites for guest molecules or protects the guest molecules and protein core from non-specific release and degradation. Moreover, the protein core could serve as a scaffold to accommodate responsive macromolecular structures that can be assembled or degraded on demand as in the case of glycogen.[79] In this scenario, a fully degradable shell could release the active protein at the target location (Figure 4b). This concept is based on the polymer-masking-unmasking-protein therapy (PUMPT) approach introduced by Duncan.[84] Here, conjugation of a biodegradable polymer to a protein protects it and masks activity in transit, while enabling controlled reinstatement of activity at the target site by triggered degradation of the polymeric component.[84]

The first generation of polymer core-shells mainly consisted of multiple conjugations of PEG chains, which have been found to effectively increase the hydrodynamic radius of proteins, thus reducing renal clearance and immune cells activation.[80] However, as a consequence of the statistical PEGylation of the proteins, a reduction of protein activity was often observed. To avoid this, site-directed conjugation of PEG further away from the active site was accomplished as discussed in section 2.1. Another evolutionary strategy involved designing polymer shells that were cleavable in target tissues to release the active form of the proteins. Recently, several customized biodegradable linkers have been developed for the cleavage of PEG chains from proteins, most of which were based on intramolecular activated esters[81] or thioesters[30] that readily hydrolyze *in vivo*. Several therapeutic proteins with biodegradable PEG shells have been accomplished and are already under *in vivo* investigation.[81-83] For instance, the cleavable PEGylated mesothelin targeted immunotoxin SS1P for the selective treatment of pancreatic carcinoma, ovarian cancer, and malignant mesotheliomas[82] has been reported recently. Immunotoxin SS1P was conjugated with three strands of branched 24 kDa PEG statistically attached at lysines via a bis-*N*-2-hydroxyethylglycinamide linker which hydrolyzed rapidly in plasma.[82] The PEGylated SS1P before hydrolysis was shown to be inactive in cell based cytotoxicity assays. In contrast, superior bioactivity was observed *in vivo* when compared to either unmodified SS1P or non-cleavable PEGylated counterparts.[82] Antitumor activity was also tested in nude mice bearing A431-K5 human cancer cells with the PEG-SS1P conjugates showing reduced toxicity, improved efficacy and markedly reduced immunoreactivity with human anti-SS1P antibodies derived from clinical samples.[81] Similarly, interferon-β-1b (IFN-β-1b) with two to three copies of cleavable PEGs[83] was studied and the mice based *in vivo* assessment revealed maximum concentrations of the circulating bioconjugates that were about 50–100 fold greater compared to unmodified IFN-β-1b at the same dosage, whereas the time of maximum concentration was delayed by four to seven fold.[83] Aside from conjugation of polymers with cleavable linkers, it is also attractive to use naturally biodegradable polymers construct a polymer core-shell system. Duncan et al.[84] have proposed a synthetic strategy to conjugate the biodegradable polysaccharide dextrin onto active proteins, such as trypsin. They showed that the protein activity was reduced by 34–69% for different dextrin sizes and degrees of modification, but it almost completely recovered after incubation with α-amylase. Although direct conjugation of polymers ("*grafting to*" strategy) is efficient to accomplish a polymer shell with diverse polymer types, the density of the polymer shell is still low due to poor conjugation efficiency of large polymer chains. In contrast, *in situ* polymerization at the surface of bioactive proteins ("*grafting from*" strategy) allowed the formation of a dense and uniform polymer shell that protected and silenced protein activity before release. Ouyang[85], Lu[86, 87] and Tang's [88] groups have independently developed approaches to grow a polymer shell on a protein surface by controlled free radical polymerization such as RAFT[89] and ATRP.[90] Vinyl initiators were firstly conjugated onto the protein surface by acrylylation[85] and the subsequent addition of monomers and *N,N,N',N'*-tetramethylethylenediamine/ammonium persulfate in aqueous solution initiated the *in situ* polymerization of a uniform shell (Figure 4a). By varying the concentration of monomers and polymerization time, the thickness of the polymer shell was controlled between 2 to 5 nm.[85] Furthermore, by varying the respective monomers, the net charge of the polymer shell was adjusted and the desired biodegradability was introduced by incorporating enzymatically cleavable peptides as crosslinkers.[86] Remarkably, in some cases, *in situ* polymerization significantly improved the stability of proteins at elevated temperature or even in the presence of organic solvents without affecting the enzymatic activities of the encapsulated proteins.[86] Several proteins have been delivered into cells via this approach with high efficiency while maintaining low toxicity, including enhanced green fluorescent protein (EGFP),[86] horseradish peroxidase (HRP),[86] bovine serum albumin (BSA),[86] superoxide dismutase (SOD),[86] caspase-3 (CAS)[86] and organophosphorous hydrolase.[87]

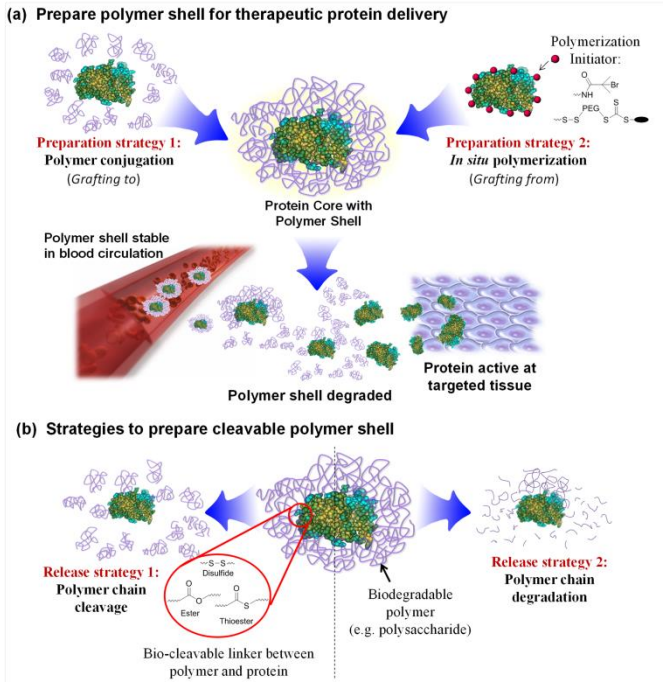

Figure 4. a) Delivery of active proteins with a polymer shell prepared by both "grafting to" and "grafting from" strategies. b) Cleavable polymer shell prepared by (1) using bio-cleavable linker between polymer and protein and (2) using biodegradable polymers.

Besides using proteins as the bioactive entities, the protein core could also serve as polypeptide scaffold to transport guest molecules and exploit the additive effects of both the polymer shell and the protein core. In nature, transporter proteins such as HSA offer defined 3D structures, uniform nanometer sizes as well as high biocompatibility. The albumin family to which HSA belongs to comprises naturally occurring transport proteins in blood responsible for lipid trafficking. They are one of the most widely used transporter proteins due to their abundance in nature, their non-toxicity and available binding pockets for loading different guest molecules.[91] Their natural functions are thus exploited in the design of albumin-polymer hybrid transporters to various cargoes including drugs and genes, often with improved loading capacity and stability due to the polymer shell compared to the native protein.[92] The conjugation of cationic polymers onto albumins facilitated efficient cellular uptake and additionally, complexation with negatively charged DNA for gene delivery.[93] Additionally, the distribution of these cationic polymers over the larger hydrodynamic radii of the protein yielded reduced cytotoxicity compared to many synthetic cationic polymers of the similar charges or size.[30, 94] As such, Segura et. al. prepared BSA-poly(dimethylamino) ethyl methacrylate (PDMA) nanoparticles by *in situ* growing of PDMA on BSA via ATRP.[94] These conjugates were shown to bind plasmid DNA (pDNA) to form nBSA/pDNA polyplexes and these polyplexes were able to transfect cells with similar efficiencies or better compared to linear, branched PEI and PDMA, with comparatively lower cytotoxicity.[94]

3.4 Protein-Dendron Core-shell Architecture

Although conventional polymers have been successful in shielding both therapeutic proteins and protein transporters, the polydispersity of polymers reduces structural precision of protein therapeutics at the molecular level and increases the risk of unpredictable side effects.[5] As such, the development of dendritic core-shell protein hybrids can be easily extrapolated and its architectural significance stems from the high volume to molecular weight ratios of these perfectly branched macromolecules.[95] Davis' group showed that the attachment of a carbohydrate based dendron onto a glycoprotein protease facilitated the anchoring of the hybrid construct onto bacterial cell surfaces.[96] Subsequent cleavage of the membrane proteins by the glycoprotein protease inhibited the infectivity of the bacteria, Actinomyces naeslundii, with an IC_{50} = 20 nM.[96] Through this approach, dendronized proteins emerged as a novel class of hybrid materials exhibiting synergistic biological properties while simultaneously maintaining molecular definition.[96, 97]

In this respect, Weil and co-workers recently reported the construction of a dendritic core-shell hybrid protein by covalently attaching generation two poly(amido)amine (G2 PAMAM) dendrons onto a human serum albumin (HSA) core via the Huigen 1,3-dipolar cycloaddition reaction.[30] The resultant dendronized HSA hybrids (HSA surrounded by second generation PAMAM) were non-cytotoxic and used as drug delivery carrier to incorporate higher amounts of the drug doxorubicin compared to native HSA, which was attributed to the additive number of binding cavities from the conjugated dendrons those of the protein core. Consequently, the G2-HSA-doxorubicin construct was evaluated to be much more potent (IC_{50} = 0.77 μM) compared to the HSA-doxorubicin construct (IC_{50} >12 μM) or the free drug (IC_{50} = 29.1 μM) towards mammalian cells.[30] The incorporation of dendrons in these systems provided additional prospect of carrier property enhancement, for instance, via functionalization of the terminal ends with targeting groups which will allow selective delivery towards cancer cell lines which overexpress certain receptors.

4. Supramolecular Protein-Polymer Hybrids

4.1 New Advances in Non-Covalent Conjugation Strategies

Covalent conjugation techniques have been represented as the dominant strategy for the construction of biohybrid conjugates, especially for the preliminary evaluation of their biological behavior and the investigation on polymer induced features. Inspired from Nature's biosynthesis of proteins, non-covalent chemistry can complement the existing covalent approaches by introducing an additional level of complexity and provide a platform for creating functional higher ordered structures. Specifically, self-assembly based on synthetic molecular recognition has developed rapidly to create protein-polymer hybrid materials with sophisticated higher-ordered architectures providing an additional avenue of macromolecular engineering.[98] The designated supramolecular functionality can serve as a ligation tool between the protein and polymer or included within the polymer to direct nanoscopic assemblies into particles,[99] vesicles/micelles,[100] protein cages[101] etc.[102]

Following the success of covalent PEGylation of proteins, it is not astonishing that supramolecular PEG conjugates were one of the first explored concepts to attach polymers through a synthetic non-covalent approach. Inspired by metal ion affinity chromatography, Pasut's group incorporated nitrilotriacetic acid (NTA) onto the terminal ends of a PEG star polymer to facilitate copper promoted binding towards histidine rich proteins such as granulocyte colony stimulating factor (G-CSF), insulin and interferon α-2b.[103] Analysis via surface plasmon resonance revealed that the binding affinity between the NTA derivatized

PEG star and G-CSF was 4.7 nM, but the expected increase in circulation half-life of the construct was not observed *in vivo* due to possible non-specific interactions with plasma proteins.[103] On the other hand, Berkland and co-workers grafted a linear PEG (5 and 20 kDa) onto pentosan polysulfate (PPS), a polyanionic polymer, to induce electrostatic binding towards positively charged keratinocyte growth factor-2 (KGF-2).[104] Consistent with results for covalent PEG conjugates, the supramolecular construction of PEGylated proteins revealed increased thermal stability and higher hydrodynamic radii.[104]

Recent interests in exploring various synthetic groups to promote supramolecular interactions have expanded the arsenal of chemical tools, with major advancement in host-guest and boronic acid based chemistries.[105-107] Scherman and coworkers utilized the specificity of macrocyclic cucurbit[8]uril towards electron deficient viologen and electron rich naphthalene to form a BSA-cucurbit[8]uril-PEG ternary complex.[15] The ability of the macrocycle to select a complementary guest promotes the ternary formation and provides an elegant supramolecular ligation approach in an equimolar fashion.[15] On the other hand, Weil's group recently adopted the high affinity between aryl boronic acids and salicyl hydroxamate moieties to assemble a pH responsive dendritic shell surrounding an enzymatic core (trypsin, papain, deoxyribonuclease I).[108] The supramolecular dendritic system acts as an activity switch through steric constraints, preventing access of biological substrates at near neutral pH (7.4). Upon acidification to pH 5.0, the disassembly of the hybrid construct released the enzyme and corresponding recovery of activity was observed. Coupled with the cationic character of PAMAM dendrons, the target enzyme was shielded, internalized into mammalian cells, and released in the acidic intracellular compartments. The discrete characteristics of such a responsive dendritic hybrid responding to these biological microenvironments represented a unique platform for the design of protein nanotherapeutics.

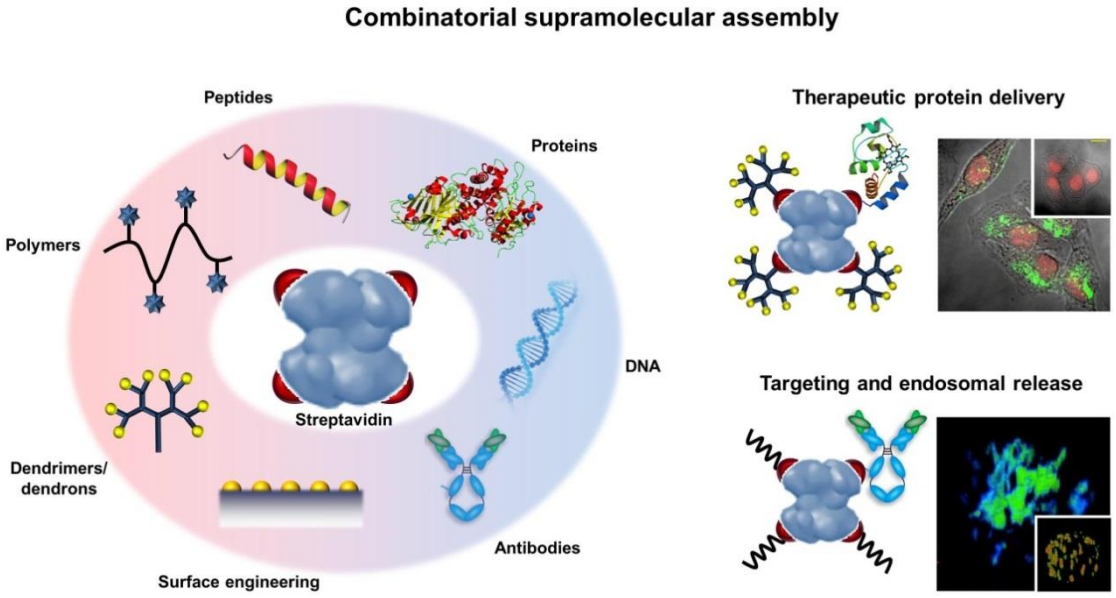

Figure 5. Versatility of streptavidin as a macromolecular adaptor for combinatorial assembly of hetero-conjugates and additive features due to the presence of both synthetic and protein entities: Enhanced cellular uptake of p53[117] as well as cell targeting and endosomal release of the biohybrid macromolecules[118].

4.2 Supramolecular Conjugates based on Streptavidin

In addition to availability of various chemical surface functionalities, proteins have been exploited as an avenue for supramolecular assemblies stemming from the specific binding interactions with their substrates. These binding sites are designated in Nature for catalysis, transportation, signalling pathways and higher ordered protein-protein interactions representing the transient and dynamic networks of biological processes. Streptavidin, a biotin-binding protein that arranges in a tetrameric configuration, has since been used as a steadfast model to create defined supramolecular constructs due to its high specificity for both biotin and its analogues. The flexibility in streptavidin-biotin binding capacity facilitated synthetic modification of biotin onto a variety of molecules and surfaces which have propagated applications in antibody therapeutics,[109] peptide/protein immobilization,[110] protein polymers[111] and drug delivery (Figure 5).[112] Intuitively, the covalent core-shell strategies explored in the aforementioned section can be directly transferred onto this supramolecular platform, eliminating the need for conducting chemical reactions on the protein.

Specifically, polymer-streptavidin systems have been extensively studied by Stayton's group beginning with a seminal study using thermo-responsive poly(N,N-diethylacrylamide) to shield and regulate the interactions between large biotinylated proteins with streptavidin.[113] Building upon this concept, several polymer-streptavidin systems have been developed for affinity separation, bio-sensors and diagnostic applications due to the robust binding conditions and stability of the protein.[114] To further exemplify this versatility, Maynard and co-workers showed, following a *grafting from* approach, an *in situ* ATRP of poly(N-isopropylacrylamide) using streptavidin as a macroinitiator to construct a stoichiometrically defined polymer-protein conjugate.[115]

These synthetic strategies were subsequently realized to be an excellent platform for supramolecular design of chemical protein therapeutics, drawing parallel to that of antibody-streptavidin conjugates developed by synthetic biology methods.[20] Several groups have shown that streptavidin can be empowered to cross cellular membranes through the conjugation of cell penetrating functionalities such as peptides,[116] membrane disrupting polymers or poly(amido)amine dendrons.[117] Utilizing a HIV-

derived peptide sequence (TAT) that promotes cellular internalization, Stayton's group showed that the TAT-streptavidin protein construct retains the ability to bind and deliver biotinylated alkaline phosphatase into mammalian cells (Jurkat, NIH 3T3).[116] In addition, activity assays of the internalized alkaline phosphatase displayed a conserved enzymatic profile directly providing an optimistic outlook for this facile supramolecular functionalization strategy.

Recent advancement has focused on the combinatorial aspect of streptavidin-biotin interaction to assemble multifunctional synthetic and bioactive macromolecules specifically addressing key biological mechanisms (Figure 5). For instance, a ternary complex composed of a biotinylated antibody, streptavidin, and biotinylated pH-responsive poly(propylacrylic acid) (PPAAc) was prepared, which offered cell specific targeting and efficient endosomal release due to the disruption of the endosomal membrane by PPAAc (Figure 5).[118] The flexibility of this multi-domain aspect was further exemplified through an assembly of biotinylated generation two poly(amido)amine (PAMAM) dendrons for cellular uptake, a biotinylated therapeutic protein of interest and streptavidin acting as a bio-adaptor.[117] These positively charged dendrons facilitated efficient cellular entry without compromising carrier toxicity up to 100 μM, providing an alternative chemical strategy towards protein delivery. In this way, tumor suppressor p53 and cytochrome C were efficiently delivered into various cancer cell lines (A549, SAOS, HeLa) and their improved activity profiles in inducing apoptosis presented an attractive prospect in protein based cancer therapeutics.[117] However the strong biotin association energy with native streptavidin strictly limits its use for biomolecules that are not hampered by this sterical restriction. To address this limitation, iminobiotin, an analogue of biotin,[119] has been applied as pH-sensitive linker that allows the controlled and reversible assembly and intracellular release of cargo molecules in acidic intracellular compartments.[87]

The pH dependency of iminobiotin derives from its guanidyl group in place of an urea functionality in biotin and the protonation state of this moiety regulates the binding affinity of iminobiotin towards both streptavidin and its mammalian counterpart, avidin.[119] Iminobiotin has been widely used in affinity purification of streptavidin fusion proteins utilizing a basic binding buffer (pH > 7.4) and an acidic elution conditions (pH < 5).[119] Intuitively, this property of iminobiotin is particularly relevant in constructing responsive supramolecular protein assemblies in a modular fashion. Utilizing an iminobiotin functionalized solid phase platform, the adaptor module (avidin) is first bound onto the surface under basic conditions allowing the diametrically opposite binding site of the protein to be exposed.[87] A macromolecular transporter consisting of a core-shell dendronized HSA mono-functionalized with a biotin tag is subsequently bound onto the exposed site followed by the elution of this bi-molecular construct at pH 4. As a consequence, the binding site previously occupied by the solid phase is now free to interact with various iminobiotinylated protein cargos with β-galactosidase and botulinum derived C2I toxin showing pH dependent release profiles.[87] This stepwise supramolecular construction of a hetero-trimeric hybrid assembly has provided a unique strategy in creating fusion proteins through chemical methods, instilling a new repertoire of possibilities into strept(avidin)-biotin based systems.[87]

4.3 Protein-Polymer Micelles and Nanoparticles

For higher ordered supramolecular architectures, self-assembly of amphiphilic polymers into micelles is one of the most widely used strategies to prepare drug delivery systems.[120] Such systems benefit from rapid preparation, high drug loading capacity, easily accessible decoration and the potential to introduce stimuli responsiveness.[121] This approach has since been adapted towards protein-polymer hybrids to create biomimetic giant amphiphiles in an effort to provide new insights towards the way these macromolecules dictate the supramolecular assembly.[122]

Although the preparation of polymer-based micelles has been well established, the self-assembly of protein-polymer hybrids into micellar drug delivery carriers is still challenging to achieve, mainly due to the synthetic difficulties in attaching hydrophobic polymers onto hydrophilic proteins and the insolubility of the hydrophobic polymer in aqueous solutions. Utilizing an expressed protein ligation technique, which exploits the self-splicing intein to produce a recombinant C-terminal thioester, Olsen's group conjugated a diblock copolymer (poly(dimethylacrylamide)-*b*-poly(*N*-isopropylacrylamide)) onto GFP in a site specific fashion.[123] The resultant hybrid reversibly self-assembled into micelles in a thermo-responsive manner (25 °C: R_h = 5.5 nm, 50 °C: R_h = 13.7 nm) thereby demonstrating that the polymeric appendage is directing the assembly process (Figure 6). To further understand whether the supramolecular structure observed is independent on the protein, two structurally homologous but electronically different proteins, mCherry and EGFP, were conjugated with a single pNIPAM chain.[124] In this study, both mCherry and EGFP are fluorescent proteins that have been genetically engineered with different electrostatic surface patchiness in order to demonstrate the broad applicability of a polymer induced self-assembly. While both materials perform identically below the thermal transition temperature, mCherry-pNIPAm formed hexagonal phases while EGFP-pNIPAm assembled into micelles above the thermo-responsive temperature.[124] The observations suggest that the electrostatic map of the target protein influences the micellar stability but other properties such as solubility, second virial coefficients and zeta potential are largely similar between the two constructs.

In addition to self-assembled micelles, protein-polymer conjugates could also be fabricated into nanoparticles. Mann et. al. used the pickering emulsion technique to process BSA-pNIPAm hybrids into hollowed nanoparticles consisting of a closely packed monolayer of conjugated protein–polymer building blocks (named proteinosomes). These proteinosomes exhibit protocellular properties such as guest molecule encapsulation, selective permeability, gene-directed protein synthesis and membrane-gated internalized enzyme catalysis, which are demonstrated by successful *in vitro* gene expression of EGFP in these proteinosomes[125]. In addition, Zare et. al[126] have reported the preparation of BSA-poly(methyl methacrylate) (PMMA) nanoparticles by a nanoprecipitation method, which is relatively simple to accomplish and easy to scale up (Figure 6). Spherical nanoparticles with diameters of around 100 nm were obtained and the water insoluble chemotherapeutic drug camptothecin was encapsulated within the hydrophobic core consisting of PMMA.[126] Such protein nanoparticles possess tunable sizes and surface charges, they can be chemically modified, have attractive biocompatibilities and allow efficient cell uptake. Camptothecin-encapsulated BSA-PMMA nanoparticles revealed enhanced anti-tumor activity both *in vitro* and in animals. One could envision here that by varying the proteins and polymers, even more sophisticated architectures and interesting properties could be expected.

In addition to retaining the native globular architecture of proteins, the possibility to exploit the polypeptide backbone of denaturized proteins as source for biopolymers opens many additional possibilities. Upon denaturation of proteins, the hydrophobic amino acids in the interior of proteins are exposed. In this case, hydrophilic substituents are attached to these lipophilic polypeptide segments in which micellar formation of

these high molecular weight polypeptides derived from proteins can be observed. With this strategy, He et al.[127] have conjugated multiple hydrophilic PEG chains onto the lysine side chains of denatured BSA. The resultant protein-polymer conjugates spontaneously self-assembled into micelles that were stabilized by the hydrophilic PEGs at the periphery. Subsequently, high numbers of hydrophobic drug molecules, e.g. camptothecin, were absorbed into the protein core to achieve biocompatible drug delivery particles[127] (Figure 6). However, micellar stability in blood circulation to prevent undesired drug leakage might still represent a concern due to the amphiphilicity of the BSA-derived polypeptide chain.

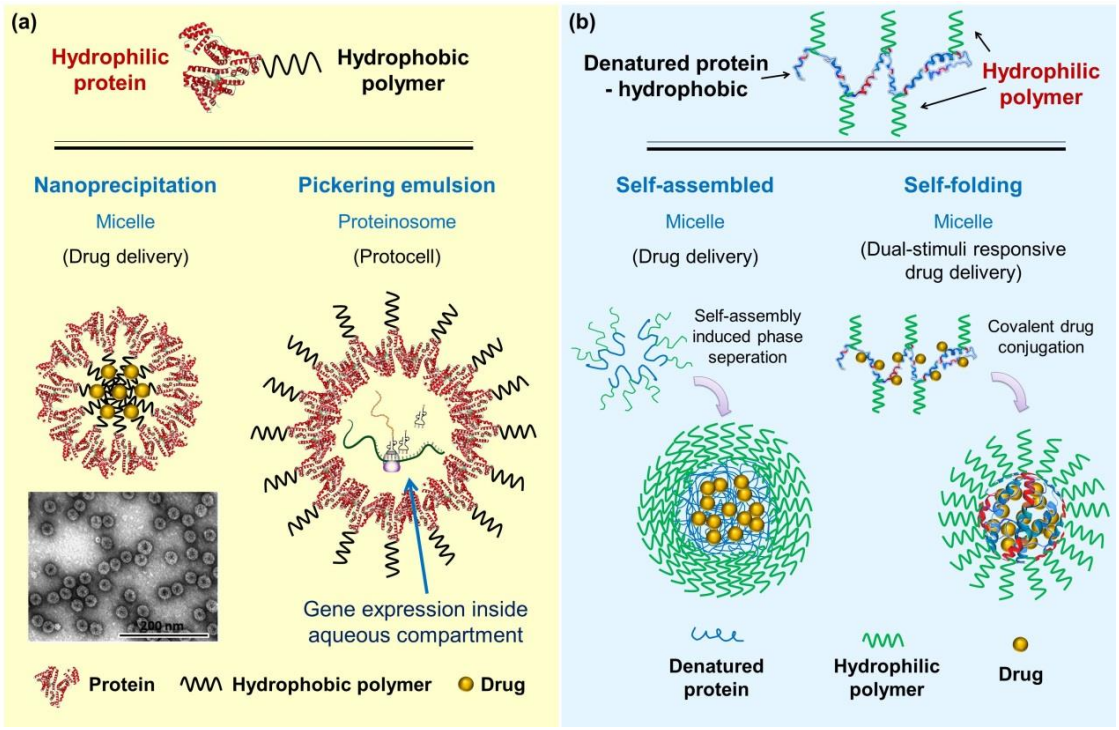

Figure 6. Giant protein-polymer micelles for drug delivery. [125-128]

To alleviate this problem, Weil et. al.[128] have reported a covalent conjugation of multiple lipophilic anti-cancer drug molecules (doxorubicin, DOX) by an acid sensitive hydrazone linker to the cysteines of the polypeptide backbone further increased hydrophobicity of the polypeptide chain. Backfolding of the albumin chain into very stable micelles was observed that was induced by the hydrophobic amino acids of HSA as well as the lipophilic DOX molecules. This supramolecular architecture exhibited a highly controlled, two-step drug release mechanism that responded to the presence of a proteolytic and an acidic environment, which only coexists inside cells. A highly potent toxicity effect in acute myeloid leukemia (AML) model was observed with IC_{50} at the sub-nanomolar range, and long term suppression of AML growth was also observed *in vivo*. In addition, many reactive groups along the amino acid side chain are in principle available for further modifications, thus offering the attachment of further targeting and bioimaging groups. Collectively, polypeptides derived from denatured proteins offers a versatile and easily accessible platform to access sophisticated protein-polymer conjugates as adaptable, multifunctional drug delivery carriers.

4.4 Protein Nanocages

Protein cages represent natural nanocarriers built from a process of controlled hierarchical assembly[129]. They usually contain a limited number of subunits, which assemble into chains or ribbons that can result in the formation of ''porous'' nanospheres (Figure 7). From a drug delivery perspective, protein cages are prominent transporters as their uniform dispersity facilitates loading of a discrete number of drug molecules whereas standard polymeric micelles possess a greater statistical variation.[130] Structurally, protein cages can be formed in a variety of shapes, each providing a different surface that, with genetic modification, equipped with bioorthogonal groups allow a broad range of chemical reactions to introduce drugs, imaging agents or fluorophore labels (Figure 7).[131, 132] In addition, their intrinsic high stability in most physiological environments effectively protects internalized drugs from enzymatic degradation.[133, 134] Furthermore, protein cages possess the capability to assemble or disassemble under controllable conditions, thus facilitating convenient cargo loading and release. In view of these attractive features, numerous applications of protein cages have been demonstrated including the delivery of imaging molecules[131, 132], drugs[133, 134], antibodies[135] and therapeutic proteins[136].

However, the application of protein cages for drug delivery is often limited by their immunogenicity, broad biodistribution as well as significant function and property variations.[137] As a result, the attachment of polymer chains to the protein cage surface has been proposed to devise a polymeric stealth layer with tunable solubility and end-functionality while maintaining structural integrity.[138] Several virus based protein cages have thus allowed investigation of the effects of PEG modifications to shield immunogenic epitopes on the surfaces and optimistic results have been obtained.[139-141] Chemically, these polymer chains have been attached following both *grafting from* and *grafting to* approaches, reiterating the stability of these virus like particles towards chemical reactions.[138] The *in situ* growth of polymer chains from the virus capsids was pioneered by Finn's and Douglas' group. Oligo(ethylene glycol)-methacrylate (OEGMA) and its azido-functionalized analogue have been

demonstrated[138] by performing ATRP from the outer surface of the bacteriophage Q β virus like particle (VLP) (Figure 7) yielding narrowly dispersed, high-molecular-weight polymers that cover the particles uniformly. Small-molecule imaging agents, such as the MRI contrast agent Gd-DOTA, and chemotherapeutics, such as doxorubicin, were introduced onto the reactive azido residues of the polymer shell.

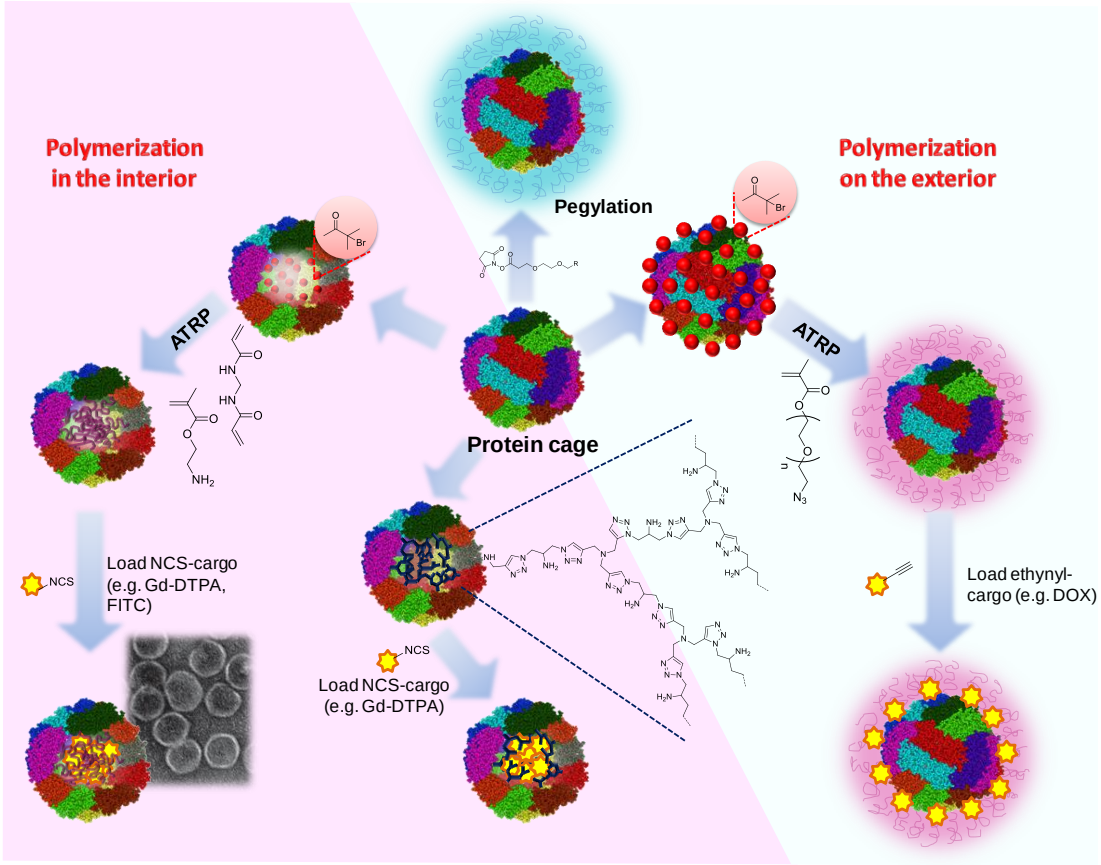

Figure 7. Summary of different methods for preparing protein cage-polymer conjugates. Polymers could be conjugated at the exterior or within the interior of protein cages by both *grafting to* and *grafting from* strategy. Cargoes were loaded to the polymers by covalent chemical conjugation.

Interestingly, polymerization reactions within a protein cage provided a high loading density of cargo molecules and increased the stability of the cage assembly.[142, 143] In this unconventional approach, 2-poly(aminoethyl methacrylate) cross-linked with bisacrylamide was polymerized inside the cavity of the p22 virus capsid using ATRP (Figure 7).[144] With a highly dense polymer core, the protein cage promoted the anchoring of small molecular cargoes including Gd-diethylenetriaminepentacetate (Gd-DTPA), a magnetic resonance imaging (MRI) contrast agent. In this fashion, a significantly increased labeling efficiency of the virus like particle has been achieved providing an atypical perspective of the potential of such multimeric protein–polymer conjugates as MRI contrast agents.[143] The increase in stability as a result of polymerization of the cage interior was demonstrated by introducing a branched polymer into heat shock protein (HSP) (Figure 7). The resulting protein-polymer hybrid revealed an increased thermal stability up to >120 °C and it possess up to 200 additional amines contributed by the polymer core providing an avenue for extensive chemical functionalization. Indeed, up to 159 DTPA-Gd complexes were loaded per particle, resulting in high T1 ionic relaxivitiy of 25 $mM^{-1}s^{-1}$. As a result, these macromolecular contrast agents have augmented particle relaxivities (4200 $mM^{-1}s^{-1}$) and have extremely high relaxivity densities (r1/particle volume).

Beyond using viruses and virus type particles, large multimeric proteins such as the abundant iron storage protein ferritin have emerged as attractive targets to function as defined nano-containers. Ferritin is composed of 24 identical protein sub-units that are ubiquitously present in all cell types thus conferring biocompatibility and non-immunogenecity to its conjugates. In this aspect, approaches to incorporate gadolinium complexes as imaging agents[145] or cisplatin as chemotherapeutics[146] were investigated due to the affinity of apoferritin towards metal cations. The reversible assembly and disassembly of ferritin is pivoted at pH 2, allowing the encapsulation of these molecular cargos above this pH.[147] With a 432-point group symmetry, functional groups of ferritin available for chemical modifications and grafting of polymers have been introduced in a highly regular fashion to provide precise spatial control.[148] These polymer-ferritin constructs exhibited protein resistant properties facilitating circulation within the bloodstream while reducing possible antibody interactions.[148] Additionally, *in vivo* studies of ferritin based nanotherapeutics have shown improved tumor suppression and reduced cardiotoxicity due to the exemplary biological behavior of these protein nanocages.[147] These results clearly demonstrate the great synergism evolving from the combination of supramolecular protein assemblies existing in nature and novel polymer design that can be empowered to fulfil therapeutic and diagnostic needs as well as

providing a revolutionary outlook on the future of supramolecular nanomedicine.

Conclusions

In this review, we have summarized the progress in protein-polymer conjugates for therapeutic applications. By exploiting a broad array of different frameworks based on protein and polymer precursors, diverse hybrid bioarchitectures have been derived to re-engineer protein functions in combination with polymer capabilities for biomedical applications. While PEGylated proteins still remain the standard and reference in protein-polymer conjugates, a divergent path has clearly surfaced, with new polymer design and an expanded arsenal for chemical engineering of proteins serving as the main driving force for the recent development of these macromolecular hybrids. Furthermore, the parallel development of non-covalent strategies, *videlicet*, stimulus responsive chemistry facilitated by supramolecular interaction has also emerged as a top-contender to contrive avant-garde macromolecular motifs for the customization of their functions, stability, activities and transportation capabilities. One can easily envisage that the repertoire of these hybrid materials will be greatly expanded for macromolecular therapeutics and they will still remain in the spotlight even decades after their first inception.

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