Minireviews
doi.org/10.1002/cmdc.202000412

Chem Europ
European C Societies P
Check for updates

Solid-Phase Protein Modifications: Towards Precision Protein Hybrids for Biological Applications

Seah Ling Kuan*[a, b] and Marco Raabe[a, b]

Proteins have attracted increasing attention as biopharmaceutics and diagnostics due to their high specificity, biocompatibility, and biodegradability. The biopharmaceutical sector in particular is experiencing rapid growth, which has led to an increase in the production and sale of protein drugs and diagnostics over the last two decades. Since the first-generation biopharmaceutics dominated by native proteins, both recombinant and chemical technologies have evolved and transformed the outlook of this rapidly developing field. This review article presents updates on the fabrication of covalent and supramolecular fusion hybrids, as well as protein-polymer hybrids using solid-phase approaches that hold great promise for preparing protein hybrids with precise control at the macromolecular level to incorporate additional features. In addition, the applications of the resultant protein hybrids in medicine and diagnostics are highlighted where possible.

1. Introduction

The introductionof the first recombinant protein-human insulin for the treatment of diabetes, over 30 years ago, prompted intense interest in the development of biopolymers such as peptides, proteins, and nucleic acids as therapeutics. Proteins, in particular, have received broad attention with over 130 molecules recorded with clinical approval by FDA in 2008. Notably, the global market therapeutic proteins reached $174.7 billion in 2015, and a revenue of $302.9 billion has been projected for 2026.[1,2] Some of the factors contributing to their burgeoning development includes highly specific mode of actions, biocompatibility, reduced immunogenicity, and biodegradability. The first generation of biopharmaceutics were typically native proteins or peptides, whereas the second generation is represented by chemically modified proteins, genetically engineered proteins with sequence modification or fusion proteins with improved pharmacological behavior such as stability and reduced side effects.[3,4] The most prevalent examples of chemically modified proteins are polymer-protein hybrids for cancer treatment in which the polymers are adopted as high-molecular-weight substituents to enhance pharmacokinetic parameters or to improve immunogenicity. Moving onwards, the current third generation seeks to address new administration routes and the development of formulations that can give rise to drugs with higher efficacy, exemplified by monoclonal antibodies.[3,4] Besides therapeutics, proteins have also been employed in diagnostics such as antibodies in immunoassays.[5] Evidently, the advancement in recombinant technologies and synthetic capabilities has been a major driving force for the innovation of protein therapeutics and diagnostics.

Recombinant technologies allow the modification of sequences by recoding genetic information at specific sequences and consequently, offer precise molecular information on the mutant proteins or protein chimeras that can be related to their subsequent activity.[6,7] In comparison, chemical technologies appear to offer fewer options for the new-generation biologics that require precise molecular knowledge of protein function due to a perceived lack of control over stoichiometry resulting in heterogeneous products, loss of activity with harsh reaction conditions for chemical modification, and lack of molecular precision in assembling higher-order protein assemblies.[8,9] As a result, chemical technologies give rise to greater challenges in biosecurity assessment and quality control of the multiple products present. Nevertheless, synthetic methods are highly valuable as complementary tools to genetic engineering and could be employed to overcome the limitations of the functional groups presented by 20 proteinogenic amino acids and offer the possibility to program external stimuli such as pH or light triggers to evolve the proteins beyond their natural functions.[10–12] Such strategies hold immense value for the advancement of synthetic biology and personalized medicine through the expansion of Nature's repertoire with unlimited synthetic combination.[13–15] Therefore, the development of synthetic tools that allow chemical modifications in site-directed manner and self-assembly of chemically modified proteins under mild conditions to surmount the challenges of selectivity and precision is necessary for the innovation of next generation therapeutics and diagnostics.

To this end, numerous designs have been developed over the last two decades using molecular bioconjugation reagents targeting natural and nonstandard amino acids, *in vitro* and *in vivo*, to address protein modifications in a precise manner,[16–22] as well as molecular reagents to induce defined protein assemblies that have been described in several

[a] *Dr. S. L. Kuan, M. Raabe*
Max Planck Institute for Polymer Research
Ackermannweg 10, 55128 Mainz (Germany)
E-mail: kuan@mpip-mainz.mpg.de
[b] *Dr. S. L. Kuan, M. Raabe*
Institute of Inorganic Chemistry I, Ulm University
Albert-Einstein-Allee 11, 89081 Ulm (Germany)

© 2020 The Authors. Published by Wiley-VCH GmbH. This is an open access article under the terms of the Creative Commons Attribution Non-Commercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2020 The Authors. Published by Wiley-VCH GmbH

excellent reviews.[8,9,23,24] Native chemical ligation has also been described to prepare synthetic proteins from amino acid building blocks with possibility to vary the stereochemistry and encoding of noncoded amino acids.[25,26] More recently, solid-phase (SP) approach have emerged as a contemporary approach to perform chemistry at defined protein sites and to prepare protein-protein conjugates with precise architectures through directed modification on complex protein surfaces.[10,27–29] This relatively young branch of precision protein engineering gave rise to new precision protein bioconjugates and protein-protein conjugates, which opens up new opportunities for treating challenging diseases. In this review article, we highlight some of the concepts and main solid-phase strategies to design *avant garde* protein hybrids and highlight some of their biomedical applications.

2. Solid-Phase Synthesis: Concept

Solid-phase synthesis (SPS) was pioneered by Merrifield in 1963 and is often associated with the chemical synthesis of peptides/proteins.[30] There are three components that are essential for the success and widespread application of this technology, which was awarded the Nobel Prize in 1984. They are 1) an insoluble and crosslinked polymeric material that is inert to the reaction condition; 2) possibility to link the substrate to the solid phase, which allows selective cleavage of the product from the solid support after synthesis; and 3) a chemical protection strategy that permits selective protection and deprotection of reactive groups (Figure 1).[30,31]

The orthogonal protecting group strategy on a solid phase has ultimately overcome the limitations in synthetic access through capitalizing on the chemical reactivity and binding selectivity of orthogonal protecting group strategy and requires minimum purification, thereby offering significant advantages over normal synthesis in a liquid state to achieve the synthesis of peptide chains and even proteins in a stepwise fashion.[26,30,31] The innate modularity of polypeptides, oligonucleotides, and oligosaccharides has been exploited to afford generalized platforms for their syntheses, thereby leading to an expanded access to these important classes of biomaterials and permitting widespread exploration and applications of their functional

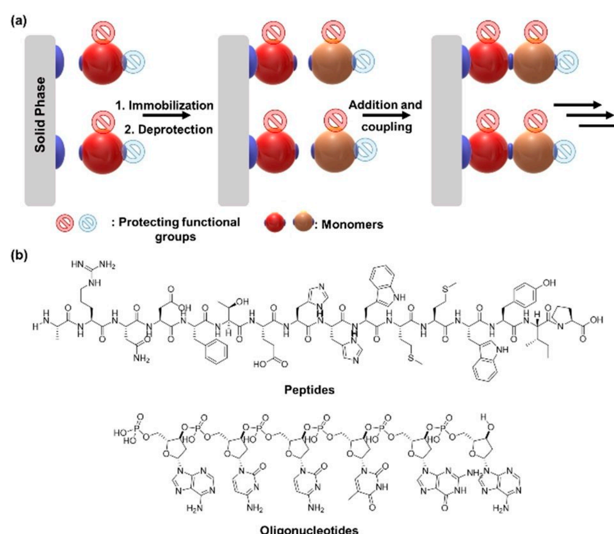


Figure 1. a) Solid-phase synthesis of oligomers by using an orthogonal protection strategy. A monomer building block with a protected functional group is immobilized onto a solid bead by covalent or noncovalent interactions, followed by selective deprotection and addition of a second building block. This process continues until the required oligomer is synthesized and cleaved from the solid support. Finally, the oligomer or polymer is purified to remove partial products and products containing errors. b) Examples of two classes of compounds prepared by SPS.

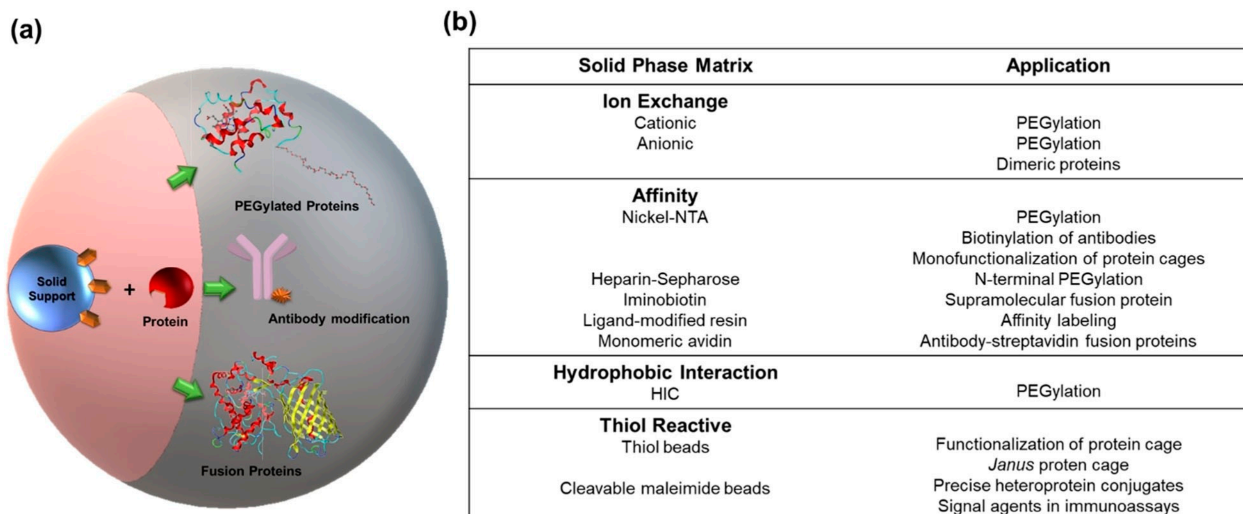
potential.[31–33] With the advancement in chromatographic technologies, there is a vast selection of solid-phase matrices that have been developed for protein purification,[34,35] and it is thus intuitive to extend SPS to macromolecules to exploit the surface masking effect provided by the solid-phase matrix for selective modification of proteins and preparation of next generation protein conjugates (Figure 2).

Protein-protein monoconjugation was reported as early as 1977 using a SP approach, but there were few advances over the following two decades.[36] Driven by increasing biomedical needs, this approach has regained attention in the last two decades and more elegant SPS strategies have since been devised to open access to a greater pool of functionalized proteins and higher-order protein conjugates for immunodetection or as nanotransporters.[5,10] The preparation of protein

Seah Ling Kuan completed her PhD at the National University of Singapore (NUS) in 2009. She joined Prof. Tanja Weil at NUS from 2008–2010 as a research fellow. In 2011, she became an Alexander von Humboldt fellow at Ulm University, becoming a group leader in the Institute of Organic Chemistry III from 2013–2016. She joined the Max Planck Institute for Polymer Research in 2016 as a group leader (Protein Therapeutics). Since 2020, she has been on the International Advisory Board of ChemMedChem. Her research focuses on developing chemical approaches for precision protein therapeutics to address diseases such as cancer, inflammation or infections.

Marco Raabe received his bachelor's (2013) and master's (2016) degrees in biochemistry from Ulm University. His master's degree in the group of Tanja Weil on "Bio-onspired Multidomain Protein Complexes by Noncovalent Self-Assembly" focused on protein modification. In April 2016 he started his PhD at UUlm before moving to the MPI-P in July 2016. His research focuses on biopolymeric coating of nanodiamonds for targeted delivery and biomedical imaging, which is part of the EU-Project Hyperdiamond (Horizon 2020).

© 2020 The Authors. Published by Wiley-VCH GmbH



Solid Phase Matrix	Application
Ion Exchange	
Cationic	PEGylation
Anionic	PEGylation
	Dimeric proteins
Affinity	
Nickel-NTA	PEGylation
	Biotinylation of antibodies
	Monofunctionalization of protein cages
Heparin-Sepharose	N-terminal PEGylation
Iminobiotin	Supramolecular fusion protein
Ligand-modified resin	Affinity labeling
Monomeric avidin	Antibody-streptavidin fusion proteins
Hydrophobic Interaction	
HIC	PEGylation
Thiol Reactive	
Thiol beads	Functionalization of protein cage
	Janus proten cage
Cleavable maleimide beads	Precise heteroprotein conjugates
	Signal agents in immunoassays

Figure 2. a) Examples of protein hybrids prepared by using SPS; b) Overview of commonly used solid-phase matrices and their applications in designing protein hybrids.

conjugates by a SP approach offers several advantages in terms of ease of purification and quality control of the product due to formation of single species. The recent progress in engineering proteins for therapeutic applications and diagnostics is highlighted in the following sections.

3. Solid-Phase Assisted Protein Functionalization

Post-translational processes found in nature allow the modification of peptides/proteins in a predefined manner, altering the functions, structures, and activities of bio-macromolecules in a highly controlled fashion.[37,38] Scientists have been inspired by these biochemical processes to install such modifications through reactions at single amino acid sites on complex protein surfaces, which eventually led to the emergence of synthetic mimicries.[23,39] The implications from site-directed conjugation that allows preservation of activity and structure are far reaching for applications including the poly(ethylene glycol) (PEG)ylation of therapeutic proteins to promote accumulation in tumor cells or reduce immunogenicity, the production of materials with novel properties, and probing the mechanism of pathological enzymes.[23,40,41] Most of these reactions are carried out in solution phase and although largely successful, one of the major disadvantage is the challenge of separation of modified from unmodified protein due to the small difference in molecular weight.[16,42] In this context, the concept of SPS developed by Merrifield can be extended to serve a twofold purpose, namely providing a surface masking effect, thereby allowing reactions at controlled reaction sites on the protein and the separation of unreacted reagents and side products from the desired products.[30,31]

3.1. Solid-phase protein PEGylation

Following the seminal report on the effect of polymer conjugated neocarzinostatin (SMANCS) in tumor tissues, which established the phenomenon widely known as the enhanced permeability and retention effect, PEGylation of proteins represents a common approach in which the polymers are adopted as high-molecular-weight substituents to improve pharmacokinetic parameters. For instance, it enhances the accumulation of the proteins, as well as address the inherently poor stability, high immunogenicity, and antigenicity of proteins, which are the major drawbacks of protein therapy.[43] The multiple lysine residue ε-amino groups together with N-terminal α-amino groups of proteins are often exploited for the random PEGylation using PEGylation reagents (e.g., succinimidyl carbonate monomethoxy poly(ethylene glycol)[44]).[45,46] The PEGylation reaction is normally carried out in the solution phase and consequently, produces a mixture of mono-, di- or multi-PEGylated proteins. Statistical PEGylation in solution could affect the activity of proteins adversely due to conformational change or blocking of the active site, and site-specific PEGylation is hence more desirable.[47] To overcome this problem, free cysteines in proteins are often exploited to achieve PEGylation in a site-specific manner as there are normally only one or two free cysteine residues per protein, and they can also be incorporated by recombinant technologies.[47] Intuitively, a SP approach could provide a greater control of the specificity of PEGylation to address targeted sites on the proteins, and this could be extended to a larger class of proteins than addressing single sulfhydryl groups. Proteins adsorbed onto a solid phase have a smaller surface exposed for modifications due to the hindrance provided by the solid-phase matrix and hence a lower PEGylation rate. Proteins bound to the column close to the active center are PEGylated only on the opposite face, thus preventing reactions

© 2020 The Authors. Published by Wiley-VCH GmbH

near the active site (Figure 3a). Several solid-phase matrices commonly employed for PEGylation are ion exchange,[48–50] heparin sepharose,[51,52] nickel affinity,[53] and hydrophobic interaction (HIC) resins.[54] Proteins successfully PEGylated using the SP approach include recombinant and native members of the fibroblast growth factor (FGF) family,[51,53,54] hemoglobin,[48] recombinant interferon α-2a,[49] human serum albumin (HSA),[50] staphylokinase (SAK),[50] recombinant human keratinocyte growth factor,[52] lysozyme,[54] enhanced green fluorescent protein (eGFP),[55] and immobilized protein cages.[56]

Capitalizing on the multiple surface charges of the native proteins, ion-exchange resins were employed in the earliest examples of SP PEGylation to adsorb the proteins of interests onto a suitable cationic or anionic column, and thereafter, a circulating solution of PEG reagent is applied through the column over a defined time.[48–50] Upon conjugation, the bound PEG shields the protein resulting in a weaker interaction with the resin, and a separation of PEGylated and native proteins is thus feasible using a linear-gradient elution. In contrast to PEGylation in solution, no further purification is necessary. Using this approach, PEGylation of hemoglobin, an iron-containing oxygen-transport metalloprotein, was achieved by reaction of the lysines on the exposed face with succinimidyl carbonate monomethoxy poly(ethylene glycol).[48] While liquid phase PEGylation led to a mixture of mono-, di-, and multi-PEGylated hemoglobin, solid-phase showed consistent degree of PEGylation with six PEGs per protein for PEG5 000 and five PEGs per protein for PEG10 000 and PEG20 000 due to the hindrance conferred by the SP matrix. Notably, the O_2 affinity of solid-phase PEGylated hemoglobin was higher than the conjugate achieved by PEGylation in solution. On the other hand, mono-PEGylation was achieved with HSA and SAK in 37 % and 45 % yield, respectively, with preservation of protein structures.[50] There was no direct comparison made to the conjugates achieved with solution phase modification but the authors observed a 50 % reduction in activity compared to the

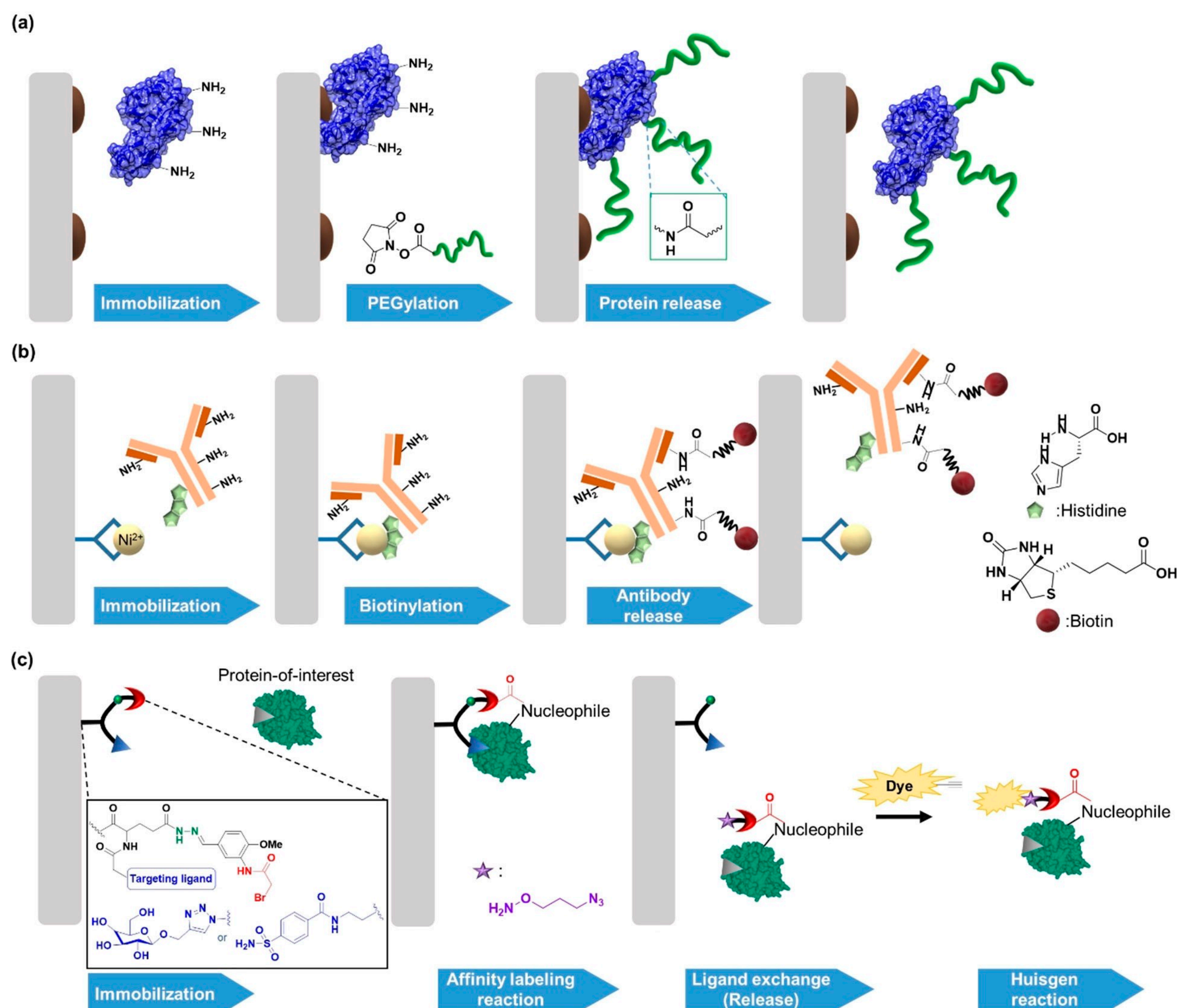


Figure 3. a) General scheme showing SP PEGylation of proteins on a defined face of proteins and b) spatial-selective biotinylation of antibody on SP. Adapted with permission from ref. [64]. Copyright: 2004, Wiley-VCH. c) Target selective modification of functional proteins by solid-phase affinity labeling. Adapted with permission from ref. [66]. Copyright: 2014, Royal Society of Chemistry.

© 2020 The Authors. Published by Wiley-VCH GmbH

native protein. In these instances, there is no clear association of the structure with the reactivity and thus the control over the degree of modification is limited. Moreover, the PEGylation of proteins on ion-exchange matrix is pH-dependent and cannot be applied to proteins sensitive to pH. Consequently, HIC and Ni–NTA affinity has also been adopted as alternatives which are independent of pH. For example, the PEGylation of lysozyme and FGF-1 on HIC was achieved.[54] Interestingly, this HIC-based PEGylation offers greater flexibility through variations of the immobilization, determined by the hydrophobicity index compared to the mPEG-butyraldehyde (mPEG) chain used, thereby broadening the range of proteins that can be modified.

For greater control of the degree of modification, SP N-terminal PEGylation was developed to produce mono-PEGylated proteins in a more systematic fashion. The guiding principle adopted here is to capitalize on the suppressed reactivity of the lysine residues in reducing alkylation conditions (e.g., at low pH).[45,57] As a result, only the most reactive amine at the N terminus react. Although N-terminal PEGylation (or modification) had been achieved in solution, rigorous purification steps were required and SP strategy is valuable to eliminate the tedious purification process.[57] Lee et al.[49] achieved an N-terminal mono-PEGylation of recombinant interferon α-2a under reduced alkylation conditions using the established ion-exchange matrix method. The mono-PEGylation was corroborated by mass spectroscopy and Edman degradation, revealing stability of the PEGylated protein against degradation by trypsin or towards temperature. However, a decrease in protein activity was observed. An alternative method was reported by Huang et al.[51] using FGF2, a member of the heparin binding growth factor family, through a heparin-sepharose SP matrix. FGF2 bound heparin on the column was PEGylated by a circulating flow under reductive conditions, followed by separation of the PEGylated and non-PEGylated proteins using ion-exchange chromatography. Although FGF2 contains 13 lysine, a high mono-PEGylation of almost 60 % was achieved with the heparin-sepharose SP, highlighting the selectivity of the approach. The method can also be extended to the N-terminal mono-PEGylation (40 % yield) of recombinant keratinocyte growth factor 1.[52] The thus PEGylated proteins revealed almost no loss of protein activity, preservation of the protein conformation, as well as a higher stability against proteolytic digestion and higher *in vivo* half-life, thus offering greater advantage over the ion-exchange SP. Nevertheless, this method is only applicable for heparin binding proteins.

The advent of microfluidics reactor technology has enabled rapid reactions, increased selectivity, and less stringent demands for reactants such as concentration and low volumes, which holds immense promise for chemical reactions not easily achieved.[58] More recent development has seen the integration of microfluidic technology for protein modification.[59] In this context, microfluidic reactors were reported for solid-phase PEGylation of the protein enzyme trypsin[60] and the enhanced green fluorescent protein, (eGFP).[55] In the former, trypsin was immobilized on magnetic particles and PEGylated.[60] After the reaction finished, the particles were separated using a magnetic gradient. The degree of PEGylation could be tuned by the reaction time and concentration of PEG applied. Notably, the PEGylated trypsin exhibit higher activity than the unconjugated protein. Nevertheless, due to a lack of physical separation of each steps in the procedure, complex reactions and cascades could not be achieved. To address this limitation, a microfluidic reactor was developed in which an organic solvent immiscible with water acts as a separation plug for compartmentalization of the different reactions.[55] Consequently, the setup allows a two-step PEGylation using 1) an amine-reactive crosslinker, disuccinimidyl suberate, to react with the immobilized eGFP, followed by 2) addition of PEG-NH_2 to achieve mono-PEGylation. Reaction paths with up to seven steps were achieved, which could be useful for semi-automated cascade reactions with proteins of interest and could be used for combinatorial screening of reaction conditions for protein modification.

3.2. Site-selective modifications of antibodies and functional proteins

Antibodies (Abs) are one of the most important class of biopharmaceutics and have found applications in both immunoassays and immunotherapy.[61–63] They are highly attractive anticancer therapeutics due to their long serum half-lives and their high binding specificity and affinity to a broad range of molecules. For the purpose of immunoassay, they are often labeled with detector molecules such as labels for fluorescent detection, bioluminescent markers, enzymes or epitope tags for immunodetection of antigen through immunohistochemistry, ELISA or western blotting, or can be used to prepare an affinity support for antigen purification.[63] However, chemical modifications of Abs in the solution phase suffer from many drawbacks including dilution and loss of Ab during purification steps, with limited recovery of the Ab. A solid-phase Ab biotinylation was developed by using the affinity of histidyl clusters in mammalian IgG-class antibodies for nickel IMAC (immobilized metal affinity chromatography) supports.[64] Here an Ab is immobilized on a nickel-chelated chromatography support and derivatized on-column (Figure 3b). After reaction, excess reagents are washed away easily, and biotinylated IgG molecule is obtained with mild elution conditions. The SP derivatization is applicable to purified IgG from all mammalian species, as the cluster of histidine residues in the Fc region is highly conserved. However, this technique is only appropriate for purified IgG molecules while SP modification of Abs in serums or other heterogeneous samples has not yet been achieved.

Although the application of site-selective modification using SP to prepare precise protein conjugates have largely been limited to PEGylation and antibody modification, such strategies clearly offer several advantages that can be expanded to other functional proteins such as enzymes to address current challenges in site-selective protein modification and purification. With this in mind, there have been further development of several methods in the last five years.[65–67] For instance, an affinity-based solid-phase approach was developed for target-selective isolation and modification of proteins such as peanut agglutinin and carbonic anhydrase II (Figure 3c).[66] A solid-

© 2020 The Authors. Published by Wiley-VCH GmbH

phase resin was immobilized with a trifunctional chemical tool comprising of 1) a ligand moiety that binds to the protein of interest, 2) an alkylating group that reacts with amine groups on the targeted protein and 3) a ligand-exchange site, that is, a hydrazone group, to release the labeled protein from the solid phase and at the same time, incorporate a reactive biorthogonal tag, such as an azide group. The purification can be easily achieved by washing steps on the solid support to remove unreacted reagents. Using monosaccharide-lectin affinity binding, peanut agglutinin was modified with 70% loading in the first steps and 52% ligand exchange in the final steps.[66] The reaction was highly selective even in a mixture of proteins containing peanut agglutinin, bovine serum albumin, ribonuclease A, and fetuin. By changing the binding ligand from β-d-galactoside to benzenesulfonamide, the method could be adopted for site-selective incorporation of an azide moiety to the carbonic anhydrase II, a protein enzyme that catalyzes reversible hydration of carbon dioxide. Notably, the site-selective modification occurs in complex biological media for example, in cell lysate of human red blood cells. Post-modification was further achieved by Huisgen 1,3-dipolar cycloaddition to include a fluorescent dye, fluorescein.

In another study, a reactive diazonium group derived from a dianiline with a *meta*-substituted CF_3 group was incorporated onto an agarose-based resin.[67] In this way, proteins containing surface-accessible tyrosine group were captured onto the solid phase and underwent diazotization. Subsequent washing and release from the solid phase was achieved under mild dithionite conditions. Proteins that consist of surface accessible tyrosines such as ribonuclease A and hen's egg white lysozyme gave high conversion rates ranging from 71–93%. Notably, a functional *o*-aminophenol was available upon release from the solid phase. This allowed further conjugation, for example, a fluorescent aniline was coupled to the modified ribonuclease A with $K_3Fe(CN)_6$ by oxidative coupling. The recent development of SP protein modification allowed target-selective isolation and modification of proteins of interest, which holds immense promise in understanding biological functions and for the development of new protein therapeutics. These methods could be potentially useful for instance to address unknown target receptor proteins of biologically active small molecules but also extended to antibody modification, to develop new antibody-drug conjugates, which are of high therapeutic relevance.

4. SPS as a Versatile Platform for Preparing Precise Protein Oligomers

The majority of proteins found in nature exist permanently as oligomers with precise spatial orientation and play significant roles in biological functions; this suggests that protein biopolymers with higher order nanostructures could be attractive therapeutic candidates.[68] Nevertheless, the complexity of protein surfaces has hindered the rational design of protein nanostructures. The advent of DNA recombinant technologies has allowed for the convenient preparation of protein chimeras comprising of two domains from individual protein species to be fused and applied in biotechnology, exemplified by antibody-fluorescent fusion proteins.[69,70] But it often suffers from the drawback of low expression levels, insoluble protein due to misfolding or loss in biological function due to wrong orientation of the protein entities or C termini modification.[70] Chemical approaches such as chemically induced homo- and hetero-dimerization were developed to overcome the disadvantages,[71] and in addition offers the possibility to instill responsive behavior that reacts to the microenvironment in the diseased cells.[10,12] Despite tremendous progress over the past decade, the preparation of protein-protein conjugates still requires strategies using concentrated protein solutions to ensure an efficient conjugation. In addition, synthetic strategies have to be devised on a case-by-case basis and tedious purification is often required to remove large polymeric by-products or unreacted materials.[72] Hence, a SP platform that requires minimal synthetic and purification processes for the preparation of precise semi-synthetic bio-nanoarchitectures will be of immense value for macromolecular therapeutics and to date, both covalent and noncovalent SP strategies have been reported to achieve this.

4.1. Covalent protein oligomers

The earliest report of employing SP for protein-protein conjugates was achieved by Pillai and co-workers using a polysaccharide matrix and glutaraldehyde as a crosslinker. By immobilizing a low density of a glycoprotein enzyme on a succinylated lectin matrix, an invertase-antimyoglobin IgG complex for high-sensitivity immunoassays was prepared.[36] Several other protein-protein monoconjugates were obtained in this fashion but are mainly limited to glycoproteins. Russell et al.[5,73] applied a modified thiol reactive SP to gain access to a series of defined protein conjugates (Figure 4a). Briefly, agarose beads are oxidized and modified to introduce a pH-cleavable maleimide linker.[73] Thereafter, a sulfhydryl-modified core protein is bound to the modified solid support, followed by the alternating addition of subsequent "layers" of sulfhydryl-modified proteins and maleimide in a sequential manner. The final protein conjugate is then recovered through cleavage of the pH-sensitive linker holding the core to the SP support. The concept to build up the protein conjugate applied here is very similar to that of layer-by-layer assemblies and in this fashion, tri-components protein conjugates consisting of R-phycoerythrin (RPE) as the core, several layers of alkaline phosphatase (AP), and a final layer of Ab was obtained in 50–76% yield through sequential maleimide-thiol reactions.[73] Remarkably, the conjugates exhibit multiple activities due to the individual protein components. The thus-assembled RPE–AP–Ab protein complexes exhibited greater uniformity in size and shape from atomic force microscopy (AFM) and gel permeation chromatography (GPC) characterization than conjugates prepared in solution-phase which showed broad size distributions.[73] The well-defined protein conjugates were subsequently applied as

© 2020 The Authors. Published by Wiley-VCH GmbH

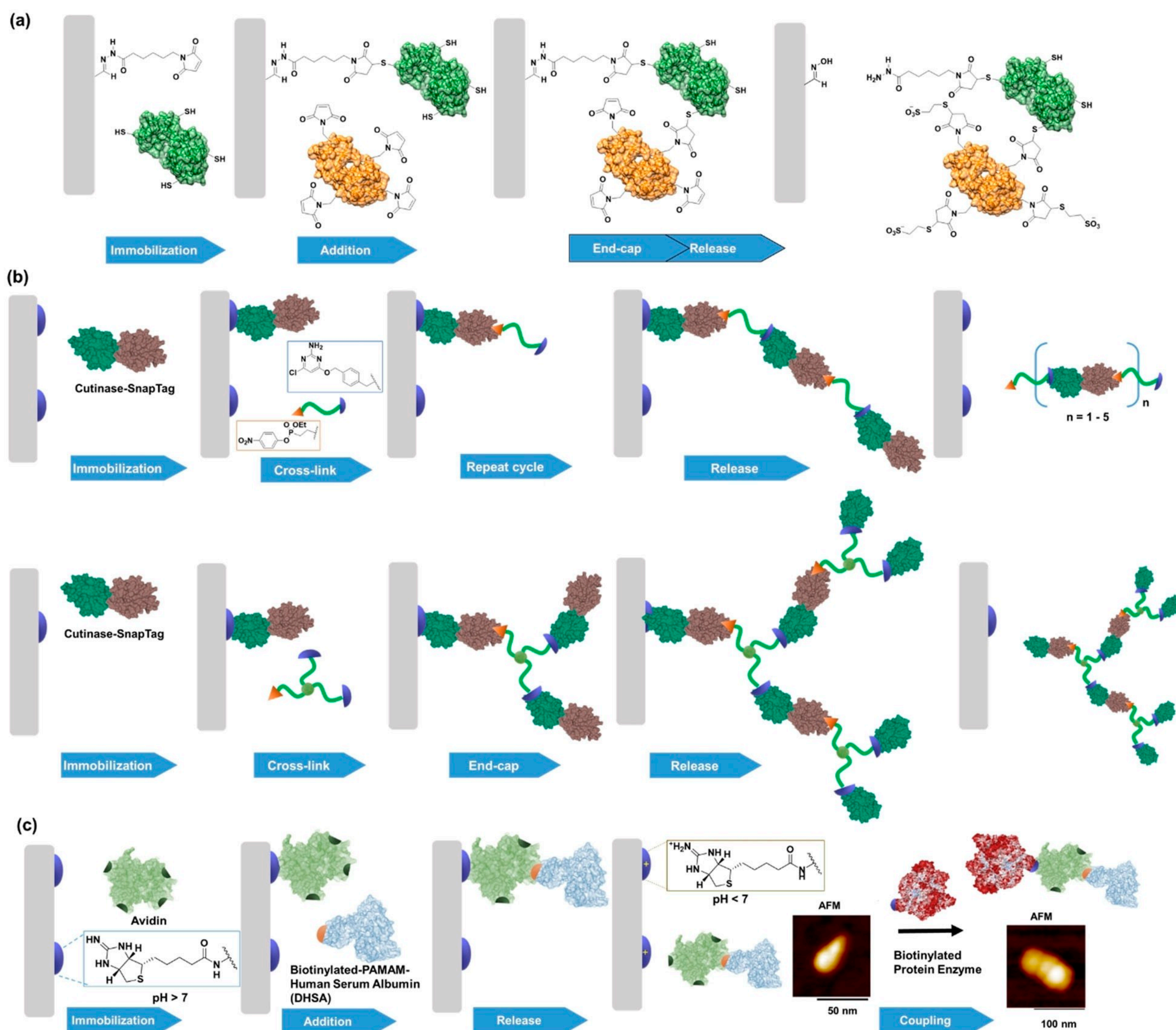


Figure 4. Summary of selected protein conjugates discussed in Section 4. a) SP preparation of R-phycoerythrin-alkaline phosphatase conjugates. Adapted with permission from ref. [73]. Copyright: 2002, American Chemical Society. b) Linear and branched multiprotein megamolecules from a fusion cutinase-SnapTag protein on SP. Adapted with permission from ref. [28]. Copyright: 2020, American Chemical Society. c) Functional heterotrimeric protein complex from a SP approach that exploits the pH-sensitive iminobiotin–avidin interaction. Adapted with permission from ref. [10]. Copyright: 2013, American Chemical Society.

in vitro immunodiagnostic reagents targeting thyroid stimulating hormone.[5] There is, however, a limitation to this approach as it does not allow the repeated use of the solid phase since it exploits the same chemistry for both conjugation and protein immobilization.

Hemoglobin (Hb)-based oxygen carriers are used as blood substitutes to address oxygen deficiency due to ischemia in various clinical settings. Stroma-free Hb solutions have been used as oxygen therapeutics but typically suffer from tetrameric dissociation, short intravascular retention time, high colloid oncotic pressure, and oxidative toxicity, thus impeding its efficacy. To overcome these limitations, size enhancements are often made through reactions with glutaraldehyde, PEG or dextran. Hb polymerized by glutaraldehyde in aqueous solution often leads to uncontrolled polymerization and multiple aggregates of varying composition, ranging from two to six Hb tetramers are obtained. Using an ion-exchange matrix, dimeric Hb tetramers were formed predominantly on the SP, which can be easily separated from the unreacted monomer.[74] Subsequent O_2 binding studies indicate that the dimers still retain their activity and exhibit lower viscosity compared to commercially available polyHb due to the low polymerization degree thus making them easier to administrate. In a similar fashion, bovine serum albumin (BSA)–Hb conjugates have also been prepared by the same group with BSA serving as a low antigenicity and immunogenicity to increase the molecular dimensions of the Hb with the conjugate showing good O_2 delivery ability and a reduction in the undesired ability of O_2 release to tissues.[75] In addition, parameters such as size and shape of protein were determined to affect preparation of the

© 2020 The Authors. Published by Wiley-VCH GmbH

protein dimers, which is valuable for preparing heterodimeric proteins in the future.

Clearly, the solid-phase approach led to the preparation of protein-protein conjugates in a more defined manner. The advantage of using a solid-phase approach is also further substantiated in a report to generate sequence-defined megamolecules with precise control of their 3D architecture.[28] The formation of such defined protein nanostructures is challenging by covalent conjugation due to formation of side-products and could require tedious purification at each reaction step. The serine esterase cutinase (C) is inhibited by an ethyl *p*-nitrophenyl phosphonate (pNPP) group through esterification of the Ser120 active-site residue while SnapTag (S) with a chloropyrimidine (CP) group reacts with the catalytic Cys145 residue of the SnapTag enzyme.[28] By exploiting the chemistry unique to each protein enzyme, Mrksich et al. designed bifunctional CP–pNPP ligands and developed a solid-phase approach to prepare so-called megamolecules comprising up to five repeating units of cutinase-SnapTag fusion protein linked by a TEV protease sequence (ENLYFQG; Figure 4b).[28] Benzylguanine-functionalized agarose magnetic beads (4% crosslinked) were first treated with a cutinase-SnapTag fusion protein (CS), which afforded the attachment of the CS through the SnapTag domain to the bead.[28] Thereafter, washing was followed by addition of a bifunctional linker having one CP and one pNPP group. The free C domain then reacts with the linker, leaving a terminal CP group for a second coupling step with another CS protein. The cycle can be repeated to prepare oligomers, that is, megamolecules, of increasing length.[28] To release the product from the solid phase, a TEV protease was added for cleavage, and a monovalent SnapTag was added to cap and terminate the reaction thereafter. In this way, tetrameric and pentameric megamolecules could be prepared and isolated in high purity, and their linear structures were confirmed by transmission emission microscopy.[28] Notably, the morphology of the resultant megamolecules can be tuned by linker design. By using a hetero-trifunctional linker that contains a terminal pNPP and two terminal CP ligands as a branching scaffold, a G2 dendron megamolecule was achieved that expanded the number of active sites with no side products observed. In this way, very large yet atomically and perfectly defined multi-domain protein oligomers can be prepared which could be interesting for biomedical applications.[28]

Solid-phase approaches were integrated with flow-based reactors to prepare protein-protein conjugates to achieve semi-automation.[76] Sortase A enzyme has been used to mediate site-specific bioconjugation of a peptide or protein substrate bearing a C-terminal LPXTG peptide tag to a substrate containing an N-terminal polyglycine nucleophile. However, it is not possible to drive the reaction to completion even with high concentration of polyglycine, and there are often side reactions such as hydrolysis and dimerization.[76] To overcome this limitation, several approaches have been proposed to immobilize either sortase A or proteins of interest on a solid phase to efficiently prepare protein-protein conjugates or protein layers.[76,77] For example, sortase A was immobilized on a Ni-NTA agarose resin to carry out bioconjugation in flow-based microreactor.[76] In this way, the product from transpeptidation was immediately released from the microreactor to minimize the reverse reaction; a nucleophile with fixed concentration could be supplied constantly at low concentration and side reactions are avoided due to minimal contact time. With this approach, the N-terminal domain of anthrax toxin lethal factor could be fused to peptides such as the MDM2 binding peptide or to form a fusion protein with the β-sheet-containing human fibronectin type III domain.

Solid-phase approaches have improved the purity, ease of synthesis and purification of covalent protein-protein conjugates. With suitable linker design, the morphology of the resultant nanostructures can be controlled to a certain extent. In combination with microfluidic technology, semi-automation is also possible. This could be potentially interesting in the future for upscaling for high-throughput production and rapid screening of protein-protein conjugates for various applications.

4.2. Supramolecular fusion proteins

Noncovalent approaches are of emerging interest because mild conditions can be employed. Under such conditions, the fusion construct is able to retain its bioactivity to impart *in vivo* functionality. Moreover, noncovalent interactions are prevalent in biological processes found in nature, for instance in the molecular assembly of higher order macromolecular structures or the interactive network of biological responses in the cells suggesting that supramolecular assembly can be exploited to create functional and responsive protein-based nanomaterials with controlled disintegration. Thus, supramolecular strategies could be used to rapidly self-assemble the protein components with a high degree of control over spatial arrangement and composition;[78] this is highly attractive for the systematic construction of asymmetric supramolecular fusion protein motifs in a "built to order" fashion that are responsive to the microenvironment of diseased cells.

The strong noncovalent interaction of avidin-biotin has been exploited extensively in molecular biology and sensing, and avidin has been used as a bioadaptor for heterofunctional bioconjugates especially in cancer pre-targeting. Precise control of the resultant stoichiometry and spatial orientation of the conjugates is limited using chemical methods and despite attempts to engineer monovalent or divalent streptavidin proteins by genetic engineering to manipulate the stoichiometry, the applications are still hampered by lower binding constants[15] and a lack of spatial control. To alleviate this problem, solid-phase strategies have been devised to modify avidin bioadaptors toposelectively. Douglas et al.[79,80] used a surface-masking approach with thiol reactive beads to biotinylate the protein cage, LiDps (the DNA binding protein from *Listeria innocua*), toposelectively and thereafter coupled streptavidin to afford the Janus particle. The Janus protein cage-streptavidin platform was purified by SEC to remove unreacted cages and transmission electron microscopy imaging showed 20% of heterodimers. Thereafter, the nanoplatform was non-covalently conjugated to a biotinylated monoclonal antibody

© 2020 The Authors. Published by Wiley-VCH GmbH

(mAb) that allows targeting of the microbial pathogen, *Staphylococcus aureus* through binding to protein A expressed on the cell surface. The multifunctional nanoplatform can be easily extended to a diverse set of antibodies for modular asymmetric functionalization to target specific cell epitopes in a "plug and play" fashion. This method also allows for the dual functionalization of the surface of protein cages to modify their surface properties.

In recent years, supramolecular chemistry has been extended to SPS[81] that allows supramolecular building blocks to be anchored noncovalently and that enables programmed self-assembly in an orthogonal fashion. Noncovalent SPS offers additional advantages in that the resins can be easily regenerated and reused unlike in covalent linkage and at the same time the binding affinity is higher than that of electrostatic interactions and could be more robust. Pengo et al.[82] combined the specific noncovalent interactions between antibody-antigen and the biotin-avidin technology to develop a SP method to obtain immunoreactive molecules similar to natural antigen-immunoglobulin M (IgM) immune complexes. An antibody that is covalently bound to the solid support is used to capture the first biotinylated protein building block (PB1), followed by addition of avidin which will complex to the resin-bound PB1. Consequently, avidin bound to PB1 has one hemisphere free for further binding with a second biotinylated protein building block (PB2). There is a certain degree of control on the topology of the complex, maintaining a high surface-to-volume ratio. In this fashion, two protein complexes, human IgM–SCCA (squamous cell carcinoma antigen) and human IgM–AFP (α-fetoprotein) were prepared that are important for the diagnosis of hepatocellular carcinoma and the pressing industrial demand for biomimetics of natural immune complexes that display efficient immunoreactivity. Subsequently, Gao and co-workers[83] have developed a protein A(G,L)–PEG-streptavidin hetero-bifunctional adaptors for the convenient assembly of unmodified antibodies and biotinylated molecules into bispecific targeting ligands using a SP approach. The bispecific molecules were achieved using a combination of two commercially available solid-phase supports, namely monomeric avidin resin and human IgG agarose. The supports were selected based on the mild conditions required for elution thus preserving protein activity and compatibility for multiple regenerations in consideration of cost effectiveness. The functionality of the adaptor components were preserved according to an enzyme-linked immunosorbent assay (ELISA). High-throughput assembly of a variety of bispecific ligands and bifunctional probes with the bifunctional adaptors was demonstrated with biotinylated quantum dots (QDs) and unmodified primary antibodies that target androgen receptor (AR) for a one-step immunofluorescence assay. In addition, the bispecific CD3×Her2 ligands were prepared in this fashion and employed as molecular targets to direct effector cells such as T cells against pathogenic target cells, which is highly valuable for cancer immunotherapy since monoclonal antibodies cannot be used to direct T cells against cancer cells.

Although humanized monoclonal antibodies (mAbs) are one of the major treatment marketed for targeted cancer therapeutics, there are still significant hurdles such as limitations associated with mAbs drug types and patient relapse due to acquired mechanisms of drug resistance. To address these challenges, new routes of administration that can target cancer-dependent pathways selectively and shun resistance mechanisms are ideal. Consequently, peptides, aptamers or modification of synthetic entities such as dendrimers with targeting groups have been developed as valuable alternatives for targeted delivery of protein drugs. Although homing peptides can be genetically fused to proteins of interest for delivery, this is impossible for non-peptide/protein based targeting systems. In this context, our group[10] has been the first to report a fusion protein comprising of a chemically post-modified proteins with SPS (Figure 4c). By exploiting the pH-dependent interaction between avidin and the imine-analogue of biotin (iminobiotin), supramolecular fusion proteins are prepared that could be applied for the delivery and controlled release of active enzymes into cancer cells. Avidin was immobilized by affinity binding to iminobiotin agarose, thereby masking one hemisphere of the avidin. In this way, a synthetically post-modified transport protein, PAMAM-human serum albumin (DHSA) *'D' for PAMAM*, was conjugated to the unprotected face of avidin and cleaved off when the pH was lowered to give a heterodimeric platform that is capable of binding different biotinylated cargo proteins to form supramolecular fusion proteins in a "built-to-order" fashion. Atomic force microscopy of the purified DHSA-avidin nanotransporter showed that 89% of the species can be attributed to the dimer, clearly demonstrating the structural integrity of the construct. The Janus-like fusion proteins enable the usually membrane-impermeable cargo proteins to be translocated across cell membranes. In addition, dissociation in acidic compartments of cancer cells is facilitated by the iminobiotin linkers and the enzymatic activity of the cargo proteins, namely, β-galactosidase and the enzymatic subunit of *Clostridium botulinum* C2 toxin, were found to be preserved. A subsequent study allows fusion of the DHSA-avidin nanotransporter with the toxin enzyme, *C. botulinum* C3,[84] the only known inhibitor of Rho-A, B and C,[85] which is involved in many diseases such as cancer, cardiovascular diseases, or inflammation.[86,87] The resultant multicomponent protein complex showed enhanced uptake into A549 lung cancer cells compared to the toxin alone and Rho-inhibition was achieved, thus suggesting that this could be applied to regulate Rho activity for therapeutic applications.[84] Notably, this method offers great potential to prepare fusion proteins comprising of a chemically post-modified proteins, aptamers or other synthetic entities with protein drugs, thus holding immense value to the design of next generation therapeutics that can address challenging healthcare issues *via* synthetic customization.

5. Conclusion

The growing global demands for protein biologics and biosensing have seen a burgeoning development of technologies to improve their properties, purification and routes of administration. In this review, we have summarized the

© 2020 The Authors. Published by Wiley-VCH GmbH

progress of solid-phase protein modification in the last few decades, which is emerging as a contemporary chemical approach that can complement recombinant technologies and address the current limitations of chemical methods with formation of single species, ease of purification and preparation. By exploiting a broad array of solid-phase matrices and proteins, this versatile approach can lead to inexhaustible design of macromolecular hybrid architectures through toposelective protein modification to engineer fusion protein constructs and protein-polymer hybrids that are highly attractive for biomedical applications. While recombinant technologies are currently the preferred method for preparing protein therapeutics, the expansion of the solid-phase toolbox has clearly increased the arsenal for chemical technologies to instill molecular precision in macromolecular engineering and pave the way for addressing some of the outstanding challenges in macromolecular therapeutics.

Acknowledgements

The authors are grateful to the financial support of the Max Planck Society, Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – project number 316249678-SFB 1279 (C01) and the European Union's Horizon 2020 research and innovation program under grant agreement [no 667192] ("Hyperdiamond"). We thank Nicole Kirsch-Pietz for proofreading. Open access funding enabled and organized by Projekt DEAL

Conflict of Interest

The authors declare no conflict of interest.

Keywords: biomedical applications · protein hybrids · protein-protein conjugates · solid-phase protein modifications

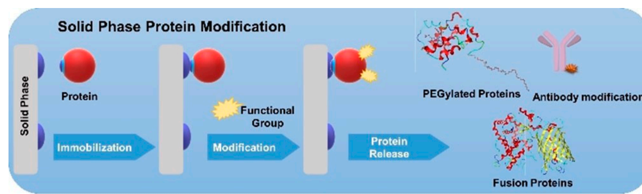
[1] "Protein Therapeutics Market: Technology Advances Spur Market Growth of Protein Therapies," L. L. Sullivan, https://drug-dev.com/protein-therapeutics-market-technology-advances-spur-market-growth-of-protein-therapies/**2019**.
[2] "Global Protein Therapeutics Market Analysis 2019," https://www.researchandmarkets.com/reports/4857806/global-protein-therapeutics-market-analysis-2019?utm_source=dynamic&utm_medium=BW&utm_code=5rr7dw&utm_campaign=1322528+-+Global+Protein+Therapeutics+Market+Study%2 C+2019+-+Market+Nears+%24303+Billion+by+2 **2019**.
[3] J. R. Kintzing, M. V. Filsinger Interrante, J. R. Cochran, *Trends Pharmacol. Sci.* **2016**, *37*, 993.
[4] J. Caravella, A. Lugovskoy, *Curr. Opin. Chem. Biol.* **2010**, *14*, 520.
[5] J. Russell, T. Colpitts, S. Holets-McCormack, T. Spring, S. Stroupe, *Clin. Chem.* **2004**, *50*, 1921.
[6] D. Filpula, *Biomol. Eng.* **2007**, *24*, 201.
[7] P. J. Carter, *Exp. Cell Res.* **2011**, *317*, 1261.
[8] O. Boutureira, G. J. L. Bernardes, *Chem. Rev.* **2015**, *115*, 2174.
[9] S. L. Kuan, F. R. G. Bergamini, T. Weil, *Chem. Soc. Rev.* **2018**, *47*, 9069.
[10] S. L. Kuan, D. Y. W. Ng, Y. Wu, C. Förtsch, H. Barth, M. Doroshenko, K. Koynov, C. Meier, T. Weil, *J. Am. Chem. Soc.* **2013**, *135*, 17254.
[11] T. Sendai, S. Biswas, T. Aida, *J. Am. Chem. Soc.* **2013**, *135*, 11509.
[12] S. L. Kuan, S. Fischer, S. Hafner, T. Wang, T. Syrovets, W. Liu, Y. Tokura, D. Y. W. Ng, A. Riegger, C. Förtsch, D. Jäger, T. F. E. Barth, T. Simmet, H. Barth, T. Weil, *Adv. Sci.* **2018**, *5*, 1701036.
[13] Y. Wu, D. Y. W. Ng, S. L. Kuan, T. Weil, *Biomater. Sci.* **2015**, *3*, 214.
[14] D. Y. W. Ng, M. Arzt, Y. Wu, S. L. Kuan, M. Lamla, T. Weil, *Angew. Chem. Int. Ed.* **2014**, *53*, 324.
[15] A. J. Heck, T. Ostertag, L. Schnell, S. Fischer, B. K. Agrawalla, P. Winterwerber, E. Wirsching, M. Fauler, M. Frick, S. L. Kuan, T. Weil, H. Barth, *Adv. Healthcare Mater.* **2019**, *8*, 1900665.
[16] M. M. Zegota, T. Wang, C. Seidler, D. Y. Wah Ng, S. L. Kuan, T. Weil, *Bioconjugate Chem.* **2018**, *29*, 2665.
[17] M. Hebel, A. Riegger, M. M. Zegota, G. Kizilsavas, J. Gačanin, M. Pieszka, T. Lückerath, J. A. S. Coelho, M. Wagner, P. M. P. Gois, D. Y. W. Ng, T. Weil, *J. Am. Chem. Soc.* **2019**, *141*, 14026.
[18] L. Xu, M. Raabe, M. M. Zegota, J. C. F. Nogueira, V. Chudasama, S. L. Kuan, T. Weil, *Org. Biomol. Chem.* **2020**, *18*, 1140.
[19] A. Maruani, M. E. B. Smith, E. Miranda, K. A. Chester, V. Chudasama, S. Caddick, *Nat. Commun.* **2015**, *6*, 6645.
[20] P. Akkapeddi, S.-A. Azizi, A. M. Freedy, P. M. S. D. Cal, P. M. P. Gois, G. J. L. Bernardes, *Chem. Sci.* **2016**, *7*, 2954.
[21] E. M. Sletten, C. R. Bertozzi, *Angew. Chem. Int. Ed.* **2009**, *48*, 6974.
[22] J. C. Jewett, C. R. Bertozzi, *Chem. Soc. Rev.* **2010**, *39*, 1272.
[23] N. Krall, F. P. Da Cruz, O. Boutureira, G. J. L. Bernardes, *Nat. Chem.* **2016**, *8*, 103.
[24] Q. Luo, C. Hou, Y. Bai, R. Wang, J. Liu, *Chem. Rev.* **2016**, *116*, 13571.
[25] S. B. H. Kent, *Protein Sci.* **2019**, *28*, 313.
[26] P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. Kent, *Science* **1994**, *266*, 776.
[27] P. Pengo, G. Veggiani, K. Rattanamanee, A. Gallotta, L. Beneduce, G. Fassina, *J. Mol. Recognit.* **2010**, *23*, 551.
[28] B. R. Kimmel, J. A. Modica, K. Parker, V. Dravid, M. Mrksich, *J. Am. Chem. Soc.* **2020**, *142*, 4534.
[29] X. Shang, D. Yu, R. Ghosh, *Biomacromolecules* **2011**, *12*, 2772.
[30] R. B. Merrifield, *J. Am. Chem. Soc.* **1963**, *85*, 2149.
[31] Z. Wang, *Compr. Org. Name React. Reagents* **2010**, 1905.
[32] D. N. Woolfson, Z. N. Mahmoud, *Chem. Soc. Rev.* **2010**, *39*, 3464.
[33] N. Hartrampf, A. Saebi, M. Poskus, Z. P. Gates, A. J. Callahan, A. E. Cowfer, S. Hanna, S. Antilla, C. K. Schissel, A. J. Quartararo, X. Ye, A. J. Mijalis, M. D. Simon, A. Loas, S. Liu, C. Jessen, T. E. Nielsen, B. L. Pentelute, *Science* **2020**, *368*, 980 LP.
[34] J. A. Queiroz, C. T. Tomaz, J. M. S. Cabral, *J. Biotechnol.* **2001**, *87*, 143.
[35] P. Cuatrecasas, *J. Biol. Chem.* **1970**, *245*, 3059.
[36] S. Pillai, B. K. Bachhawat, *Biochem. Biophys. Res. Commun.* **1977**, *75*, 240.
[37] Y. C. Wang, S. E. Peterson, J. F. Loring, *Cell Res.* **2014**, *24*, 143.
[38] G. A. Khoury, R. C. Baliban, C. A. Floudas, *Sci. Rep.* **2011**, *1*, 1.
[39] S. L. Kuan, T. Wang, T. Weil, *Chem. Eur. J.* **2016**, *22*, 17112.
[40] S. Shaunak, A. Godwin, J.-W. Choi, S. Balan, E. Pedone, D. Vijayarangam, S. Heidelberger, I. Teo, M. Zloh, S. Brocchini, *Nat. Chem. Biol.* **2006**, *2*, 312.
[41] S. Brocchini, A. Godwin, S. Balan, J. Won, J.-W. Choi, M. Zloh, S. Shaunak, *Adv. Drug Delivery Rev.* **2008**, *60*, 3.
[42] R. L. Kwant, J. Jaffe, P. J. Palmere, M. B. Francis, *Chem. Sci.* **2015**, *6*, 2596.
[43] H. Maeda, *Adv. Drug Delivery Rev.* **2001**, *46*, 169.
[44] T. Miron, M. Wilchek, *Bioconjugate Chem.* **1993**, *4*, 568.
[45] G. M. Bonora, S. Drioli, *PEGylated Protein Drugs Basic Sci. Clin. Appl.*, Birkhäuser Basel, Basel **2009**, pp. 33–45.
[46] B. Maiser, F. Dismer, J. Hubbuch, *Biotechnol. Bioeng.* **2014**, *111*, 104.
[47] N. Nischan, C. P. R. Hackenberger, *J. Org. Chem.* **2014**, *79*, 10727.
[48] X. Suo, C. Zheng, P. Yu, X. Lu, G. Ma, Z. Su, *Artif. Cells Blood Substitutes Biotechnol.* **2009**, *37*, 147.
[49] B. K. Lee, J. S. Kwon, H. J. Kim, S. Yamamoto, E. K. Lee, *Bioconjugate Chem.* **2007**, *18*, 1728.
[50] X. Suo, X. Lu, T. Hu, G. Ma, Z. Su, *Biotechnol. Lett.* **2009**, *31*, 1191.
[51] Z. Huang, C. Ye, Z. Liu, X. Wang, H. Chen, Y. Liu, L. Tang, H. Zhao, J. Wang, W. Feng, X. Li, *Bioconjugate Chem.* **2012**, *23*, 740.
[52] Z. Huang, G. Zhu, C. Sun, J. Zhang, Y. Zhang, Y. Zhang, C. Ye, X. Wang, D. Ilghari, X. Li, *PLoS One* **2012**, *7*, e36423.
[53] L. Song, Y. Zhu, H. Wang, A. A. Belov, J. Niu, L. Shi, Y. Xie, C. Ye, X. Li, Z. Huang, *Biomaterials* **2014**, *35*, 5206.
[54] J. Niu, Y. Zhu, Y. Xie, L. Song, L. Shi, J. Lan, B. Liu, X. Li, Z. Huang, *J. Chromatogr. A* **2014**, *1327*, 66.
[55] R. Fraas, J. F. Hübner, J. Diehm, R. Faas, R. Hausmann, M. Franzreb, *Biotechnol. Bioprocess Eng.* **2019**, *24*, 382.
[56] K. Uto, K. Yamamoto, K. Iwahori, T. Aoyagi, I. Yamashita, *Colloids Surf. B* **2014**, *113*, 338.

© 2020 The Authors. Published by Wiley-VCH GmbH

[57] O. Kinstler, G. Molineux, M. Treuheit, D. Ladd, C. Gegg, *Adv. Drug Delivery Rev.* **2002**, *54*, 477.
[58] K. S. Elvira, X. C. i Solvas, R. C. R. Wootton, A. J. deMello, *Nat. Chem.* **2013**, *5*, 905.
[59] R. Fraas, J. Diehm, M. Franzreb, *Front. Bioeng. Biotechnol.* **2017**, *5*, 1.
[60] K. E. Ottow, T. Lund-Olesen, T. L. Maury, M. F. Hansen, T. J. Hobley, *Biotechnol. J.* **2011**, *6*, 396.
[61] A. Beck, T. Wurch, C. Bailly, N. Corvaia, *Nat. Rev. Immunol.* **2010**, *10*, 345.
[62] S. Menard, S. M. Pupa, M. Campiglio, E. Tagliabue, *Oncogene* **2003**, *22*, 6570.
[63] I. A. Darwish, *Int. J. Biomed. Sci.* **2006**, *2*, 217.
[64] E. Strachan, A. K. Mallia, J. M. Cox, B. Antharavally, S. Desai, L. Sykaluk, V. O'Sullivan, P. A. Bell, *J. Mol. Recognit.* **2004**, *17*, 268.
[65] C. B. Rosen, R. L. Kwant, J. I. MacDonald, M. Rao, M. B. Francis, *Angew. Chem. Int. Ed.* **2016**, *55*, 8585.
[66] D. Kuwahara, T. Hasumi, H. Kaneko, M. Unno, D. Takahashi, K. Toshima, *Chem. Commun.* **2014**, *50*, 15601.
[67] C. Allan, M. Kosar, C. V. Burr, C. L. Mackay, R. R. Duncan, A. N. Hulme, *ChemBioChem* **2018**, *19*, 2443.
[68] E. N. Salgado, R. J. Radford, F. A. Tezcan, *Acc. Chem. Res.* **2010**, *43*, 661.
[69] S. K. Sharma, K. A. Chester, K. D. Bagshawe, *Handbook of Therapeutic Antibodies*, 2nd ed., Wiley Blackwell **2014**, pp. 475–486.
[70] S. R. Schmidt, *Curr. Opin. Drug Discov. Devel.* **2009**, *12*, 284.
[71] R. Kluger, A. Alagic, *Bioorg. Chem.* **2004**, *32*, 451.
[72] J. A. Modica, Y. Lin, M. Mrksich, *J. Am. Chem. Soc.* **2018**, *140*, 6391.
[73] J. C. Russell, T. L. Colpitts, S. R. Holets-McCormack, T. G. Spring, S. D. Stroupe, A. D. Vogt, S.-T. Wong, *Bioconjugate Chem.* **2002**, *13*, 958.
[74] T. Hu, D. Li, Z. Su, *J. Protein Chem.* **2003**, *22*, 411.
[75] T. Hu, Z. Su, *J. Biotechnol.* **2003**, *100*, 267.
[76] R. L. Policarpo, H. Kang, X. Liao, A. E. Rabideau, M. D. Simon, B. L. Pentelute, *Angew. Chem. Int. Ed.* **2014**, *53*, 9203.
[77] M. Raeeszadeh-Sarmazdeh, R. Parthasarathy, E. T. Boder, *Biotechnol. Prog.* **2017**, *33*, 824.
[78] R. F. Garmann, R. Sportsman, C. Beren, V. N. Manoharan, C. M. Knobler, W. M. Gelbart, *J. Am. Chem. Soc.* **2015**, *137*, 7584.
[79] P. A. Suci, S. Kang, M. Young, T. Douglas, *J. Am. Chem. Soc.* **2009**, *131*, 9164.
[80] S. Kang, P. A. Suci, C. C. Broomell, K. Iwahori, M. Kobayashi, I. Yamashita, M. Young, T. Douglas, *Nano Lett.* **2009**, *9*, 2360.
[81] A. Porzelle, W.-D. Fessner, *Angew. Chem. Int. Ed.* **2005**, *44*, 4724.
[82] P. Pengo, G. Veggiani, K. Rattanamanee, A. Gallotta, L. Beneduce, G. Fassina, *J. Mol. Recognit.* **2010**, *23*, 551.
[83] H. Y. Liu, P. Zrazhevskiy, X. Gao, *Bioconjugate Chem.* **2014**, *25*, 1511.
[84] S. L. Kuan, C. Förtsch, D. Y. W. Ng, S. Fischer, Y. Tokura, W. Liu, Y. Wu, K. Koynov, H. Barth, T. Weil, *Macromol. Biosci.* **2016**, *16*, 803.
[85] M. Vogelsgesang, A. Pautsch, K. Aktories, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2007**, *374*, 347.
[86] C. Li, H. Liu, Y. Sun, H. Wang, F. Guo, S. Rao, J. Deng, Y. Zhang, Y. Miao, C. Guo, J. Meng, X. Chen, L. Li, D. Li, H. Xu, B. Li, C. Jiang, *J Mol Cell Biol* **2009**, *1*, 37.
[87] J. H. Osaki, G. Espinha, Y. T. Magalhaes, F. L. Forti, *Oxid. Med. Cell. Longev.* **2016**, *2016*, 6012642.

Manuscript received: June 9, 2020
Accepted manuscript online: July 15, 2020
Version of record online: ■■■, ■■■

© 2020 The Authors. Published by Wiley-VCH GmbH



Dr. S. L. Kuan, M. Raabe*

1 – 12

Solid-Phase Protein Modifications: Towards Precision Protein Hybrids for Biological Applications

Molecular precision in macromolecular engineering: This review summarizes solid-phase strategies that have emerged for selective protein modification and nanofabrication of protein–protein conjugates. The applications of the resultant protein hybrids in medicine and diagnostics are also highlighted.