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Manipulating Cell Fates with Protein Conjugates

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Abstract

The homeostasis of cellular activities is essential for the normal functioning of living organisms. Hence, the ability to regulate the fates of cells is of great significance for both fundamental chemical biology studies and therapeutic development. Despite the notable success of small-molecule drugs that normally acts on cellular protein functions, current clinical challenges have highlighted the use of macromolecules to tune cell function for improved therapeutic outcomes. As a class of hybrid biomacromolecules gaining rapidly increasing attention, protein conjugates have exhibited great potential as versatile tools to manipulate cell function for therapeutic applications, including cancer treatment, tissue engineering, and regenerative medicine. Therefore, recent progress in the design and assembly of protein conjugates used to regulate cell function is discussed in this review. The protein conjugates covered here are classified into three different categories based on their mechanisms of action and relevant applications: 1) regulation of intercellular interactions; 2) intervention in intracellular biological pathways; 3) termination of cell proliferation. Within each genre, a variety of protein conjugate scaffolds are discussed, which contain a diverse array of grafted molecules, such as lipids, oligonucleotides, synthetic polymers, and small molecules, with an emphasis on their conjugation methodologies and potential biomedical applications. While the current generation of protein conjugates is focused largely on delivery, the next generation is expected to address issues of site-specific conjugation, *in vivo* stability, controllability, target selectivity, and biocompatibility.

INTRODUCTION

As the basic structural and functional module of the human body, cells play essential roles in almost every aspect of physiological or pathological processes. The homeostasis of normal functions and roles of cells is closely associated with organism health[1–5]. Thus, the control and manipulation of cell function is a major focus of biomedical research and drug development.

Although small-molecule therapeutic agents have achieved extraordinary success in tuning cell functions for disease treatment, primarily by interfering with protein function, current challenges faced by modern medicine have highlighted the utility of macromolecules as

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tools to study and manage cell behavior. Indeed, therapeutic proteins constitute a rapidly expanding class of macromolecular research tools and clinically powerful therapeutics[6–9]. Over the last decade, there has been a rapidly growing interest in the development of protein bioconjugate based drugs due in part to their high level of clinical efficacy and usefulness as research tools. Conjugation of proteins with carbohydrates, lipids, oligonucleotides, synthetic polymers, and other small molecules has been shown to alter the behavior of the protein in definable ways, especially with regard to therapeutic payload delivery, allowing the protein conjugates to achieve better synergistic outcomes.

Among clinically approved protein bioconjugates, conjugates of antibodies or antibody-derived proteins have shown the greatest clinical and diagnostic success. This class of macromolecules consists not only of IgG proteins, but also Fab fragments, single chain antibodies (scFvs), nanobodies, and other immunoglobulin-derived molecules[10]. For example, antibody drug conjugates (ADC) have been shown to be potent and specific anti-cancer agents, as well as useful for target specific radio imaging[11–13]. The success of antibody-based conjugates has been largely attributed to the targeting ability of monoclonal antibodies as cargo carriers in concert with their ability to be modified with various functional payloads. Nevertheless, there are also some limitations associated with the antibody-based conjugates, one of which is the relatively laborious, time-consuming and costly preparation process of mammalian cell-based antibody expression and purification[12,14,15]. Alternatively, investigators have also constructed a variety of non-antibody-based protein scaffolds to serve as targeting domains of protein conjugates. These scaffolds exhibit multiple advantages over antibody-based proteins, including ease of preparation, high yield, high stability, and improved safety features[9,16]; thus, they have also been exploited to generate a large number of protein conjugates for the manipulation of cell functions.

In recent years, the toolbox of engineered non-antibody protein scaffolds has greatly expanded, including, for example, affibodies, affilins, avimers, atrimers, DARPins, and Fn3s[9,16]. Using these proteins as targeting elements, an array of different protein conjugates has been generated through diverse conjugation methods. Moreover, other proteins without targeting capabilities have also been developed to serve as functional building blocks for protein conjugate construction. For several of these proteins, their self-assembling properties or favorable biocompatibility has been used to facilitate the fabrication of new supramolecular structures, as well as reversible function when encountering a specific stimuli[17–19]. All these proteins possess unique functionalities that can be used to prepare new assemblies whose properties can be tailored to fulfill distinct objectives related to the programing of cellular activities.

Here, different protein conjugates are classified into three major categories and reviewed based on their respective mechanisms to manipulate the fate of cells including 1) regulation of intercellular interactions, 2) intervention in intracellular biological pathways, and 3) termination of cell proliferation (Figure 1). Within each category, the chemical structures, conjugation methods, and specific applications of the protein conjugates are examined. Finally, a perspective of this research field is provided on the future development of therapeutic protein conjugates.

REGULATION OF INTERCELLULAR INTERACTIONS

Cell-cell interactions are vital for all multicellular organisms and serve critical roles in numerous biological activities including cell differentiation, tissue development, neurotransmission, immune responses, and cancer progression[20–25]. Although these interactions are highly dynamic in duration and complicated in the composition of cell types and mechanisms, substantial efforts have been devoted to understanding them, and the insights gained from that work have greatly advanced our ability to manipulate them for clinical treatment[26,27]. For instance, chimeric antigen receptor (CAR) T therapy relies on a genetic engineering approach to modify the T cell surface with cancer-recognizing proteins that mediate their interactions with cancer cells. Albeit efficacious clinically, these CAR-T therapies are hindered by requisite genetic engineering methods, which result in several limitations including a time-consuming preparation process, high cost, safety issues, inconsistent transfection efficiency, and toxicity by potential un-regulated immune responses[28,29]. Additionally, these genetic engineering approaches are not amenable to some cell types, such as stem cells. Therefore, investigators have sought to develop non-genetic approaches for engineering cell-cell interactions[28].

To program a pair of cell-cell interactions using non-genetic methodologies, the cell surface of the interacting pair needs to be decorated with engineered scaffolds that mediate cell-cell interactions through covalent bonding or non-covalent interactions. Thus, cell surface molecules, such as proteins, lipids, and polysaccharides, typically function as handles for non-genetic cell surface modifications. Accordingly, functional domains engaging these cell surface molecules were incorporated into the bioconjugates to induce cell-cell interactions. Here, diverse types of protein conjugates used to manipulate cell-cell interactions are described based on their chemical structures. In addition, the conjugation methodologies employed for their assembly and subsequent applications of these conjugates are also discussed.

Protein Conjugates to Regulate Intercellular Interactions

As previously noted, a number of different protein conjugates have been designed to mediate cell-cell communication through interactions with cell surface molecules, and these interactions can be generally divided into two categories according to their specificity or non-specificity. Cell surface antigens are unique biomarkers and can be specifically targeted by many types of binding molecules including proteins, oligonucleotides aptamers, and small-molecule ligands. Thus, these species can be integrated into the protein conjugates for specific cell surface binding. In contrast, plasma membrane lipids can non-specifically interact with hydrophobic moieties, such as lipids. Therefore, lipid molecules can also be conjugated to proteins and used to non-specifically tether functional proteins on the cell surface as a universal method for cell surface modifications and concomitant intercellular interactions. Moreover, protein conjugates containing reactive chemical moieties can also non-specifically react with natural chemical groups on the cell membrane or with bioorthogonal groups incorporated onto the cell surface via metabolic engineering.

Lipid insertion is a simple and direct method for cell surface modifications (Figure 2A). Protein-lipid conjugates can be generated via chemical reactions or enzymatic processes and

intercalated into the lipid bilayer of the cell membrane through hydrophobic interactions. The protein components in the conjugates normally serve as binders of cell surface antigens to mediate cell-cell interactions. For example, palmitate-conjugated proteins have been generated by the Peacock group and used as “surrogate receptors” to mediate intercellular interactions since 1989[30,31]. Others have adopted the same methodology to construct palmitate-protein G conjugates to functionalize the surface of mesenchymal stem cells (MSCs) or progenitor cells with antibodies for targeted cell delivery[32–34]. Meanwhile, chemokine receptor proteins or chemokine proteins were also conjugated to lipids and anchored on the surface of MSCs with the goal of enhancing their interactions with target tissues[35,36]. Moreover, targeting proteins such as single-domain antibodies were directly lipidated through enzymatic ligation by sortase A to regulate interactions between T cells and myeloid-derived suppressor cells[37]. Self-assembled targeting proteins were also developed and enzymatically lipidated by protein prenyltransferase to form multivalent protein-lipid conjugates for programming cell-cell interactions[19]. In addition, a non-targeting structural protein, gelatin, was also modified with cholesterol as a membrane anchor to enhance intercellular adhesion within cells of the same type[18]. Furthermore, other functional small molecules have also been conjugated to proteins to engineer cell-cell interactions (Figure 2B). For example, biotinylation, a commonly used strategy to modify proteins for various biomedical applications, was applied to antibodies and used to mediate cell-cell contacts with streptavidin as the bridging unit[38].

Polymers are also commonly used as building blocks in protein conjugates to regulate cell-cell interactions. For instance, hyaluronic acid (HA) has been frequently used as a liver-targeting molecule and can be incorporated into protein conjugates as a targeting domain. Accordingly, a hyaluronate-wheat germ agglutinin conjugate was generated to coat the MSC surface for liver-targeted stem cell delivery[39] (Figure 2C). Although some of these natural polysaccharides can be used as targeting domains, in most cases, polymers were synthesized and grafted to the proteins for the fabrication of supramolecular structures, such as hydrogels, to encapsulate a group of cells and deliver them to specific tissues (Figure 2D). These hydrogel materials function as cell carriers, to not only maintain the cell-cell interactions within the network, but also to control the in-situ interactions of the encapsulated cells with target tissues at the injection site. In these hydrogel systems, proteins were typically conjugated to the polymers to provide supportive functionalities. For instance, insulin-like growth factor-1 (IGF-1) protein or its C domain fragment was chemically immobilized to a polymeric hydrogel for targeted delivery of cardiac progenitor cells or stem cells, where IGF-1 was shown to promote the survival, differentiation, and proliferation of therapeutic cells[40–42]. Other growth factors, such as vascular endothelial growth factor (VEGF), were also conjugated to hydrogels to play supportive roles for targeted cell delivery[43]. Moreover, the extracellular matrix (ECM) glycoprotein, fibronectin, was also coupled to hydrogels to facilitate cell adhesion and spreading[44].

Although oligonucleotides have also been engineered as receptor binders for decades, they have generally been used alone to serve as bispecific cell engagers through hybridization or conjugated with lipids to induce cell-cell interactions, which have been reviewed by others[45,46]. In addition, oligonucleotides have been deployed as self-assembling frameworks for antibody-oligonucleotide conjugates to mediate cell-cell interactions (Figure 2E).

Through hybridization with different antibody-oligonucleotides conjugates, bispecific or multispecific complexes can be formed as cell engagers for adoptive cell therapies[47,48].

Conjugation Methods for Generating Protein Conjugates that Regulate Intercellular Interactions

The construction of protein bioconjugates relies on selective chemical reactions, generally with reactive groups present on amino acid side chains or other bioorthogonal groups incorporated into the proteins. Perhaps the most commonly used way to modify proteins is by reaction between primary amines and N-hydroxysuccinimide esters, which requires the appended molecules to be converted to their respective active esters for subsequent acylation. Palmitic acid has typically been activated as the N-hydroxysuccinimide (NHS) ester for the preparation of protein-lipid conjugates[30–34], and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE) based lipids can also be conjugated to proteins using this strategy[36]. These methods have also been applied for the synthesis of protein-polymer conjugates[40] or for biotinylation[38]. Additionally, maleimide-containing lipids have also been used to functionalize thiol groups present on cysteines[35]. Other functional lipids, such as cholesterol, have been coupled to proteins through the reaction between primary amines and cholesteryl chloroformate[18]. Moreover, aldehydes, carboxylic acids, and carbonyl diimidazole were also employed to react with amines in proteins to generate protein conjugates[39,42,44].

Although effective and simple, such protein modification methods also have disadvantages with a major issue being that these reactions are not site-specific and therefore may produce heterogeneous conjugate products. Furthermore, if a nonspecific modification site is near the ligand-binding domain of the protein, the conjugated molecules may exhibit decreased function rendering them less useful for mediating cell-cell interactions[31]. To solve these problems, bioorthogonal groups, such as azide groups, have been used for click reactions with alkyne-containing molecules[41], and aldehyde-containing unnatural amino acids have also been site-specifically incorporated into proteins to react with aminooxy-modified peptide nucleic acids (PNA)[48]. Moreover, highly efficient and site-specific enzymatic reactions have also been employed to synthesize protein conjugates. For instance, the ligation reaction catalyzed by sortase A has been used to construct protein-lipid conjugates[37]. The naturally occurring lipidation reaction, protein prenylation, has also been employed to synthesize protein-lipid conjugates for the regulation of cell-cell interactions[19]. Multiple biorthogonal functional groups for preparing more complex chimeric protein structures have been installed using this approach[49]. Compared with conventional chemical modifications, these enzymatic reactions are more efficient in their ability to generate homogenous site-specific conjugate products. They also avoid the use of toxic organic solvents, which is more compatible with biomedical applications involving mammalian cells.

Applications of Protein Conjugates that Regulate Intercellular Interactions

Although protein-conjugates were originally synthesized for fundamental studies of cell surface engineering and cell-cell interactions[18,30,31], the great potential of these hybrid materials has inspired investigators to use them for cell-based therapies. One of the

most common applications of protein conjugates in the field of cell-based therapy may be regenerative medicine, involving the delivery of stem cells or progenitor cells. Protein-lipid conjugates were made for the targeted delivery of MSCs to inflammatory human vascular endothelial cells[34] or the delivery of chondrocytes to cartilage injury sites for cartilage repair[32]. Moreover, growth factor-conjugated hydrogels were synthesized to deliver adipose-derived MSCs for the treatment of acute kidney injury[41], the treatment of limb ischemia[42], and chondrogenesis[50]. Protein-HA conjugates were also generated for the systematic delivery of MSCs to the liver for liver disease treatment[39]. Furthermore, cancer immunotherapy greatly relies on the ability to control immune-cancer cell-cell interactions, which can be modulated by protein conjugates. To this end, various lipidated proteins and protein-oligonucleotides conjugates have been constructed to regulate cell-cell interactions between cytotoxic T cells and specific cancer cells, which were shown to induce cancer-specific killing by surface-engineered T cells[19,37,47,48]. Cell-based therapies have now demonstrated notable success in multiple clinical fields, including tissue engineering, regenerative medicine, and cancer immunotherapy[51]. These protein conjugates have highlighted their utility in controlling intercellular interactions of therapeutic cells and therefore have been regarded as valuable tools for biomedical applications.

INTERVENTION IN INTRACELLULAR BIOLOGICAL PATHWAYS

Apart from the regulation of intercellular interactions, protein conjugates can also be engineered to alter cell fates by manipulating intracellular biological pathways. These effects can be generated mainly by two means including the targeted intracellular delivery of functional oligonucleotides to change gene expression and the control of signal transduction through receptor binding and activation. For the first category, functional oligonucleotides can be either conjugated to a targeting protein to form protein-oligonucleotide conjugates or non-covalently carried by protein-polymer conjugates. Both approaches for oligonucleotide delivery aim to interfere with gene expression and finally disrupt the production of target proteins within the cell. Alternately, cellular signal transduction that is essential for normal cell function can be regulated by protein conjugates that act on surface receptor-mediated pathways. Although toxin delivery by protein-drug conjugates can also be regarded as an approach to block some intracellular biological pathways, in general the goal has been to broadly interrupt the essential pathways for cell survival, particularly cancer cells. Therefore, toxin delivery by protein conjugates is discussed in a subsequent section. In the next section, protein conjugates used for oligonucleotides delivery and surface receptor activation are reviewed with a focus on their structures, conjugation methodologies, and particular applications.

Protein Conjugates to Regulate Gene Expressions

It has been two decades since oligonucleotides were approved by FDA and used as targeted therapeutics, and since then, a wide variety of types of nucleic acid drugs have been developed, including antisense single-stranded DNA (ssDNA), small interfering RNA (siRNA), small hairpin RNA (shRNA), messenger RNA (mRNA), and plasmids[52]. Aside from lipid particles and virus-based vectors, engineered protein conjugates have also been commonly used to specifically deliver oligonucleotides to their target cells[53].

As an emerging class of chimeric biomolecules, antibody-oligonucleotide conjugates (AOC) have been used for many bioanalytical applications, such as immuno-PCR (iPCR), proximity ligation assays (PLA), electrochemical proximity assays (ECPA), and microscopy[54]. For therapeutic purposes, functional oligonucleotides can be delivered by protein conjugates through covalent bonding or noncovalent interactions (Figure 3A). For instance, an siRNA designed to silence the peptidyl-prolyl cis-trans isomerase B (PPIB) gene has been conjugated to a variety of antibody-based proteins targeting different cancer-related antigens, including TENB2, Steap1, EtBr, NaPi2b, HER2, MUC6, and mesothelin[55]. The siRNA for the hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene was also covalently conjugated to an anti-CD71 Fab for targeted delivery to cardiac and skeletal muscle[56]. Meanwhile, antisense oligonucleotides targeting the MAX dimerization protein 3 (MXD3) gene or FAM107A gene were also conjugated to different antibodies for anti-cancer gene therapies[57,58]. Alternatively, protamine or other positively charged polymers have been conjugated to various monoclonal antibodies to serve as carriers of negatively charged therapeutic oligonucleotides through electrostatic interactions. Antibodies targeting several common cancer-associated surface antigens, including HER2, EGFR, and PSMA, have been utilized to deliver siRNA payloads designed to knock down gene expression of essential cellular proteins[59–65].

In addition to antibody-based targeting domains, other protein scaffolds have also been employed for conjugation with therapeutic oligonucleotides. For example, several functional expression plasmids were non-covalently loaded onto biotinylated transferrin protein through biotin-streptavidin bridging and were specifically delivered to human erythroleukemia cells expressing transferrin receptors[66]. Human serum albumin was also used as a vehicle to deliver RGD-modified oligonucleotides to integrin-expressing tumor cells[67]. Moreover, CRISPR-Cas9–mediated precise genome editing has shown great potential for gene therapy. To improve the efficiency of such genome editing, site-specific Cas9-oligonucleotide conjugates were developed to recruit the donor DNA template to the target site and were shown to markedly increase homology-directed repair efficiency in both human cell culture and mouse zygotes[68,69]. Similarly, Cas12a-crRNA conjugates were also generated using bioorthogonal chemistry with site-specifically modified Cas12a proteins and 5’ chemically modified crRNA for genome editing in CAR-T cells[70].

Protein Conjugates to Regulate Signaling Transductions

Signal transduction, or cell signaling, is the transmission of biomolecular signals from a cell’s exterior to its interior, which is mediated by cell surface receptors. Therefore, receptor-targeting proteins have often been utilized as activators of downstream receptor-mediated signaling pathways, and conjugation of additional functional fragments to those proteins can function to optimize their efficacy. For this purpose, protein-oligonucleotides conjugates have been produced to activate cell signaling pathways, where the oligonucleotides serve as the framework of nanostructures for the multivalent display of receptor-activating proteins (Figure 3B). For instance, the clinical vaccine immunogen eOD-GT8 protein was conjugated with DNA origami nanoparticles for B-cell receptor activation and the nanoparticles were shown to drive functional B-cell responses[71]. Similarly, bispecific or multispecific complexes were formed through the hybridization of antibody-oligonucleotides conjugates

and shown to effectively activate T-cells for anti-cancer cytotoxcicity[47,48]. Moreover, the multimerization of protein-oligonucleotides can also be conducted on the cell surface and was demonstrated to effectively trigger the clustering of CD20 on B cells and activate downstream apoptosis signals[72]. In addition to oligonucleotides, photo-switchable molecules can also be incorporated into receptor-binding proteins to reversibly control their conformation, receptor binding and activation. For example, the photo-switchable reagent 3,3'-bis(sulfonato)-4,4'-bis(chloroacetamido)azobenzene (BSBCA) was conjugated to an anti-EGFR repebody for light-driven control of receptor binding, and downstream signaling[73].

Moreover, recent success in small molecule proteolysis targeting chimeras (PROTAC) has also inspired the development of antibody-conjugated PROTACs for the site-specific delivery of the protein degradation molecules to desired tissues (Figure 3C). For example, several PROTAC molecules degrading bromodomain-containing protein 4 (BRD4) have been conjugated to the antibodies targeting a number of cancer-specific antigens, including C-lectin-like molecule 1 (CLL1), HER2, and six-transmembrane epithelial antigen of the prostate 1 (STEAP1)[74–77]. Meanwhile, estrogen receptor (ER) was also reported to be degraded by the anti-HER2 antibody-PROTAC conjugates as a potential treatment of ER-positive breast cancers[78]. Lysosome-targeting chimeras (LYTACs) are another emerging type of antibody-based conjugates that utilize the lysosome-trafficking receptors to degrade target proteins for the manipulation of cell signaling and have been reported to degrade several membrane-bound receptors, including EGFR, CD71, and HER2[79,80]. These protein-degrading conjugates exerted selectivity for both cell surface antigens and intracellular protein targets, effectively disrupting the signaling transduction pathways controlled by the target proteins.

Functional proteins, especially growth factors, have also been frequently coupled to polymeric hydrogels to tune the cellular functions of local tissues for regenerative medicine (Figure 3D). Transforming growth factor beta-1 (TGF-β1) was covalently conjugated to chitosan hydrogels for the treatment of articular cartilage defects[81]. VEGF was also conjugated to a temperature-sensitive aliphatic polyester hydrogel for cardiac repair[82]. In another example, fibronectin functional domains were coupled to hyaluronan to stimulate adult human dermal fibroblast responses for wound healing[83]. Apart from tissue regeneration, protein-hydrogel conjugation has also been used to tune the immune response. For example, apoptosis-inducing anti-Fas monoclonal antibodies were conjugated to the surface of PEG-modified hydrogels to actively provide localized immunosuppression of autoreactive Fas-sensitive immune cells. This immunosuppression caused protective effects on transplanted pancreatic islet cells for the treatment of type I diabetes mellitus[84]. Other types of apoptosis-inducing protein ligands, such as TNF-related apoptosis-inducing ligand (TRAIL), were also used for conjugation with functional polymeric materials to trigger apoptosis of their cognate target cells[85]. Polymers can also be used to enhance the biodistribution and targeted localization of protein therapeutics with PEGylation being the most widely used modification method for this purpose. For instance, PEGylation was applied to modify bispecific fibronectin-based protein inhibitors of EGFR and IGF-1 for the inhibition of proliferative and survival signaling in cancer cells[86], and the conjugated synthetic polymers greatly changed the pharmacokinetic properties of the modified proteins

by increasing their hydrodynamic radius and preventing the uptake of the conjugates by macrophages, which consequently enhanced the therapeutic outcomes of the conjugates. Other functional materials, such as graphene oxide, have also been conjugated with proteins and applied to modulate cell fates[87].

Conjugation Methods for Generating the Protein Conjugates that Intervene in Intracellular Biological Pathways

The preparation of most protein conjugates discussed in this section depends on endogenous nucleophilic functional groups including free thiols on cysteines and primary amines on lysines. Bifunctional cross-linkers, such as succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC)[55,81], Sulfo-SMCC[60–62], N-succinimidyl 4-(2-pyridyldithio)butanoate (SPDB)[55,56], succinimidyl 3-(2-pyridyldithio)propionate (SPDP)[67], and other reagents containing both NHS ester and maleimide moieties, have been used to link amine-containing molecules to solvent accessible cystines[47,63,64,72,81]. Moreover, enzymatically cleavable valine-citrulline-containing peptides or reducible disulfide bonds can also be integrated into these linkers for the intracellular release of oligonucleotides[56]. Maleimides[85,86] or NHS esters[82,84] can also be directly added onto polymer side chains during polymerization for subsequent coupling with functional proteins. Conversely, other functional groups can also be introduced into proteins using genetic methods that facilitate incorporation of unnatural amino acids. For example, an azide-functionalized amino acid was incorporated into recombinant Cas9 protein for conjugation with DBCO-modified oligonucleotides through strain-promoted click reaction[68]. Additionally, the noncanonical amino acid, p-acetylphenylalanine (pAcF), was incorporated into proteins and reacted with aminooxy-derivatized ligands of oligonucleotides[47] or polymers[59]. These methods ensure the site-specific conjugation of the functional moiety to the target protein, although they require a relatively complicated process to produce the protein.

Applications of Protein Conjugates that Intervene in Intracellular Biological Pathways

As previously mentioned, protein conjugates can be used for gene therapies to alter gene expression in the target cells. To date, most protein conjugates targeting gene expression have been developed as potential cancer therapeutics. Based on the targeting proteins used for the assembly of such constructs, these conjugates can specifically deliver therapeutic oligonucleotides into different types of cancer cells including prostate carcinoma[55,62], B cell acute lymphoblastic leukemia[57], glioblastoma[58], melanoma[67], colon cancer[60,61], breast cancer[59,63,64], and others. Protein-oligonucleotide conjugates have also been constructed to silence myostatin and hypertrophy of the gastrocnemius for the regulation of muscle function and the treatment of muscular diseases[56].

Through the activation of specific surface receptors, protein conjugates can also regulate the functions of immune cells for cancer immunotherapy[47,48] and enable rational design of molecular vaccines[71]. Additionally, protein-conjugates have been used to directly target cancer-specific receptors to trigger apoptosis as a potential cancer treatment[72,85,86]. Protein-functionalized hydrogels have also been synthesized as possible regenerative medicines for cardiac repair[82], wound healing[83], and treatment of articular cartilage defects[81]. Protein-

PROTAC conjugates have shown great potential as targeted therapeutics and are becoming an emerging type of ADC molecule for cancer treatment[74–78].

TERMINATION OF CELL PROLIFERATION

The protein-conjugates used to terminate cell proliferation are virtually all protein-based delivery systems for cytotoxic drugs, which are designed to completely suppress cellular function and inhibit cell division. Therefore, such molecules are typically used for cancer therapies. As one of the most successful types of targeted cancer therapeutics, antibody-drug conjugates (ADCs) have been established and used as anticancer agents for almost four decades. The global antibody-drug conjugate market size was valued at USD 4.3 billion in 2020 and is expected to grow at a compound annual growth rate (CAGR) of 23.7% from 2021 to 2028, demonstrating the expanding therapeutic potential of ADC drugs. Since the history, development, applications, marketing, and future directions of ADCs have been comprehensively discussed in prior literature[11–13,88], this section mainly focuses on non-antibody protein conjugates prepared for targeted toxin delivery. These conjugates involve several types of engineered protein scaffolds as targeting domains, with toxic drug payloads that were prepared either via covalent protein modification or by non-covalent interactions with a protein-conjugate that serves as the drug carrier.

Protein Conjugates to Terminate Cell proliferation by Toxin Delivery

In the last 20 years, more than a dozen of non-antibody-based protein scaffolds have been generated to target different cell surface receptors for research or theranostic purposes[16]. These macromolecules share several benefits over conventional IgG-derived proteins that are advantageous for the development of protein-drug conjugates for the treatment of corresponding cancers[16] (Figure 4A). For instance, an affibody engineered to bind HER2 receptors was fused to an albumin-binding domain and conjugated with the cytotoxic maytansine derivative MC-DM1 to inhibit cell division of HER2-overexpressing cancer[89,90]. In related work, the photosensitizer, IR700, was conjugated to an affibody specific for the platelet-derived growth factor receptor β (PDGFRβ) for vascular-targeted photodynamic therapy of colorectal cancer[91]. Additionally, a small and soluble nanobody was developed to target CD38, and the highly potent antineoplastic agent, MMAE, was conjugated to that anti-CD38 nanobody for the treatment of multiple myeloma[92]. DARPin proteins constitute another emerging class of protein scaffolds that can be used for targeted cancer drug delivery. Accordingly, they have been engineered to exhibit a variety of molecular sizes and half-lives with the goal of specifically delivering the cytotoxic auristatin derivative, MMAF, to EpCAM-positive cancer cells[93]. Similarly, MMAF was also coupled to an EGFR-specific repebody for targeted cancer therapy[94]. Naturally existing proteins have also been explored for their ability to deliver conjugated toxins. For example, PEGylated conjugates of fibroblast growth factor 2 (FGF2) with hydrophilic auristatin were prepared for highly selective killing of cancer cells overexpressing fibroblast growth factor receptor 1 (FGFR1)[95].

Rather than directly conjugating the drug payload to the targeting protein, the delivery of cytotoxic molecules can also be carried through non-covalent interactions. Since the

chemotherapeutic agent, doxorubicin, can intercalate into double-stranded DNA, some protein-oligonucleotide conjugates have been developed for the targeted delivery of doxorubicin to cancer cells[96,97] (Figure 4B). Radionuclides can also be loaded into protein-oligonucleotide conjugates for radiotherapy[98]. Protein-polymer conjugates have also been generated and self-assembled into nanoparticles for the encapsulation and delivery of cancer drugs[17,99] (Figure 4C). For example, bovine serum albumin (BSA) is a commonly used protein scaffold for conjugation with hydrophobic polymers, and the resulting BSA-polymer nanoparticles have been used to deliver drugs, such as doxorubicin[100] and camptothecin[101]. Likewise, silk sericin-polylactide protein-polymer conjugates have been constructed for the intracellular delivery of doxorubicin[102]. It is of note that protein-lipid conjugates can also be incorporated into liposomes as targeting elements to direct the liposomes to cancer cells for drug delivery purposes[103,104].

Conjugation Methods for Generating Protein Conjugates that Terminate Cell Proliferation

For the conjugates covered in this section, the coupling reaction between maleimides and sulfhydryl groups on proteins was the most frequently used method to generate protein-drug conjugates. Various cancer chemotherapeutics, including mertansine and auristatin derivatives have been designed and synthesized to incorporate maleimide functional groups facilitating conjugation to cysteines to a cancer-specific targeting protein[90,91,93,95]. Similarly, primary amines on proteins can also be coupled with NHS ester-functionalized drugs[91] or reacted with acrylic acid NHS ester for polymerization initialization to form protein-polymer conjugates as drug vehicles[101]. Bifunctional linkers, such as Sulfo-SMCC or succinimidyl-6-hydrazino-nicotinamide (S-HyNic) have also been utilized to crosslink free amines on proteins to thiol-functionalized oligonucleotides[96,97] or aldehyde-modified polymers[102].

In addition to non-specific lysine and thiol coupling strategies, enzymatic reactions have also been employed as site-specific conjugation methods for the assembly of next-generation protein-drug conjugates using various enzymes[94,105–108]. For instance, farnesylation was used to incorporate an aldehyde-containing farnesyl analog into an anti-EGFR repebody, followed by subsequent ligation with aminooxy-containing MMAF[94]. Also, by first conjugating the maleimide-functionalized MMAE to a cysteine-containing short peptide, the cancer drug, MMAE, was linked to a CD38-targeting nanobody through sortase A catalyzed ligation[92]. Compared with non-specific reactions, these enzymatic methodologies can produce homogenous products with high efficiency, which is more desirable for industrial pharmaceutical production.

Applications of Protein Conjugates that Terminate Cell Proliferation

Cytotoxic drug delivery by protein conjugates is primarily employed for cancer treatment, and the specific application of each protein conjugate is largely driven by the specificity of the protein component in the conjugate to a cancer-specific antigen. Therefore, the target cancer type treated by the conjugates is largely dependent on the type and expression level of the corresponding antigen. Accordingly, anti-HER2 protein conjugates were used to treat HER2-positive ovarian cancer[89,90] and anti-EpCAM protein conjugates were prepared to target EpCAM-overexpressing colon adenocarcinoma and

breast cancer[93]. EGFR-overexpressing triple-negative breast cancer, epidermoid carcinoma, and lung adenocarcinoma can be targeted with the corresponding anti-EGFR protein-drug conjugates[94,96,100]. Other cancer-associated antigens, including PDGFRβ, CD38, and FGFR1 have also been targeted to treat colorectal cancer, myeloma, and lung carcinoma respectively[91,92,95]. For some protein-polymer based nanoparticles, no specific targeting elements were involved, and the localization of the drug at tumor tissues was dependent on the enhanced permeability and retention (EPR) effect or the pH-responsive property of the protein-polymer conjugates[101,102].

Apart from cancer cells, protein-drug conjugates can also be applied to eliminate dysfunctional non-cancerous cells for therapeutic purposes. For instance, doxorubicin intercalated antibody-DNA conjugates were developed to specifically deplete Myo/Nog cells for the treatment of posterior capsule opacification (PCO) following cataract surgery because myofibroblasts emerge from Myo/Nog cells and can cause PCO in some adults and most children. The protein conjugates targeting G Protein-Coupled Receptor Kinase 1 (GRK1) were shown to effectively deliver doxorubicin to Myo/Nog cells without off-target effects[97].

PERSPECTIVE

Albeit effective in numerous pre-clinical studies and some clinical applications, a number of hurdles to their expanded clinical application remain. Hence, the development of new methods for the preparation and use of protein-conjugates is of keen interest, especially with regard to the manipulation of cell fates. In this section, the desirability and challenge of optimizing protein conjugation preparation, functionality, and clinical application are discussed.

Conjugation Methods

A hallmark of protein modification is the control of the quality of the final material by the conjugation methodology. Regardless of the conjugate composition, appropriate reactive groups on the protein are required for the attachment reaction. The most abundant reactive groups on proteins for conjugations are lysine and N-terminal primary amines. Hence, such amines have been widely used for linking lipids, oligonucleotides, polymers, and other molecules to proteins through reactions with NHS esters (Figure 5A). However, an obvious drawback of this nonspecific method is that it produces heterogeneous products, since specific lysine conjugation is difficult to achieve, resulting in difficulties in achieving product quality control. The coupling reaction between thiols (often obtained via disulfide reduction) and maleimides is another approach for preparing protein conjugates (Figure 5B). While single free thiols can be engineered into non-natural scaffold proteins, in antibodies they must be obtained by selective reduction of disulfides, a strategy that requires careful control of reaction conditions that may ultimately disrupt the structure and function of the protein, as well as enable the production of heterogeneous material, which is one of the major obstacles to the quality control of therapeutic antibody-drug conjugates. Another issue with these non-specific conjugation methods is the deactivation of functional proteins. For example, uncontrolled nonspecific conjugations may lead to protein precipitation or

unfavored masking of the antigen-binding domains of antibodies or the active sites of enzymes[31].

To overcome these limitations, site-specific conjugation reactions were developed including the incorporation of unnatural amino acids that contain bioorthogonal groups into proteins[48,59,68] (Figure 5C,D). Though viable, the preparation of such engineered proteins can be time-consuming and inefficient[109]. Therefore, various enzymatic reactions have also been exploited in recent years for site-specific protein functionalization[110] (Figure 5E-H). These enzymes, including sortase A[37], microbial transglutaminase[108], phosphopantetheinyl transferase[107], and prenyltransferase[19,94], typically catalyze conjugations in aqueous solution with high efficiency, and require only that target proteins be genetically fused with a short tag for recognition. Moreover, due to the relaxed substrate tolerance of many of these enzymes, they can often be used to transfer functional groups such as azides or alkynes that can then be employed in a broad range of subsequent chemical conjugation reactions via bioorthogonal reactions[94]. Although current clinically investigated protein conjugates have been mainly constructed using non-specific modification methods, the promising potential of enzymatic conjugation approaches will likely be further employed in the development of next-generation therapeutic protein conjugates.

Functional Proteins

For most of the protein scaffolds discussed in this chapter, their role is typically to serve as targeting domains and bind specific cell surface receptors to either mediate cell-cell interactions, induce endocytosis, or trigger receptor-mediated signaling. These molecules include antibody-derived moieties or non-antibody proteins that have affinities for a variety of cell surface antigens. For many biomedical applications with these types of protein conjugates, a major concern is how to improve their selectivity to minimize off-target side effects, especially for the delivery of toxic drugs or gene-silencing oligonucleotides. One strategy is to fabricate multivalent scaffolds with multiple receptor-specific proteins to increase the avidity of the conjugates. The multivalent scaffolds can be formed either by modifying the single conjugate with multiple targeting proteins[39] or through self-assembly processes to form supramolecular structures[19,47,48,71]. Moreover, different types of targeting domains can be incorporated into the same conjugate to generate bispecific or multi-specific constructs to enhance selectivity[86]. For protein-drug conjugates, such enhanced selectivity means greater accumulation of the drug at the tumor site and less premature drug release in normal tissues. It is expected that personalized protein-drug conjugates with multi-specificity and high selectivity can be developed based on analysis of the patient's antigen type and expression level. Furthermore, engagement of multiple receptors may also be used to boost signal transduction upon receptor activation[72], further emphasizing the significance of multivalency for designing protein conjugates.

On the other hand, temporospatial control over the functionalities of protein conjugates is also highly favorable for therapeutic purposes. For example, it would be highly beneficial if a protein conjugate can mediate reversible cell-cell interactions in a controllable manner because tunability has the potential to reduce the incidence and severity of potential adverse effects from cell-based therapies. In other words, the ability to "switch off" protein

function can provide a higher level of safety and efficacy using protein conjugates compared with other approaches designed to mediate cell-cell interactions. Nevertheless, only a few protein conjugate systems to date have permitted temporal control over their function. For example, prenylated multivalent nanoring systems have been shown to mediate reversible cell-cell interactions, which can be abolished by the treatment with an FDA-approved drug, trimethoprim[19]. These studies have highlighted the significance of function reversibility and provided insights into the design of smart controllable systems. With the increasing need to optimize the selectivity of the protein conjugates for their targets, more efforts have been put into the development of multivalent protein conjugate constructs. The interplay between the avidity and selectivity of these multivalent constructs has also been investigated, providing insights into targeted drug delivery and regulation of cell-cell interactions[111]. Furthermore, when bispecific multivalent conjugates are used to engineer intercellular interactions, the ratio of the targeting elements can be tuned based on the relative number of the two types of corresponding receptors to induce optimal interactions[19,112], and the concentration of the bispecific conjugates at their binding sites is another consideration for efficiently directing intercellular interactions according to the ternary complex model.

Functional Elements Conjugated to the Proteins

Next generation conjugates will likely depend on taking advantageous and compatible chemistry that can enhance conjugation synthesis. To regulate cell-cell interactions, protein-lipid conjugates need to be stable on the cell surface for a desired period of time. However, many protein-lipid conjugates employed for cell surface modifications and cell-cell interactions exhibit only short half-lives on cell surfaces ranging from mere minutes to a few hours. To increase the stability of protein-lipid conjugates on the cell surface, the hydrophobicity of the anchor can be enhanced for a stronger affinity with membrane lipids, or multivalent scaffolds can be constructed to increase the number of lipid anchors necessary to achieve greater stability[19].

For protein-polymer conjugates used for hydrogels, the properties of the polymeric materials are of critical importance for biomedical applications. The polymeric materials should incorporate the necessary self-assembling and rheology characteristics, biocompatibility with tissues, and, if desired, controlled biodegradability[113,114]. In addition, especially for drug delivery applications, protein-polymer conjugates may need to be engineered to be responsive to defined physiological stimuli[102]. Likewise, photo-switchable linkers can also be rationally designed and incorporated into functional proteins to control their conformations and functionalities[73].

Oligonucleotides conjugated to proteins can be designed with self-assembling properties for multivalent display of targeting proteins[47,48,71,72], and their structure can also be chemically modified to obtain better *in vivo* stability towards enzymatic degradation[72]. It is likely that the combination of multiple functional molecules within the same protein conjugate may lead to synergistic effects, enhancing therapeutic efficacy.

CONCLUSION

Protein conjugates are versatile hybrid biomacromolecules that can be used to manipulate cell fate. Different functional materials have been conjugated to proteins for a plethora of biomedical applications, including regulation of intercellular interactions, intervention in intracellular biological pathways, and inhibition of cell proliferation. Collectively, the emergence of protein conjugate techniques and methodologies has demonstrated their promising potential for therapeutic application in the fields of cell-based immunotherapy, tissue engineering, regenerative medicine, targeted gene therapy, cancer drug delivery, and others. Although the clinical translation of these molecules remains challenging, studies employing these conjugates have accelerated the development of new conjugation methods and deepened our understanding of what constitutes favorable clinical properties for “ideal” conjugates. Overall, protein conjugates are poised to play increasingly significant roles in many biomedical applications well into the future.

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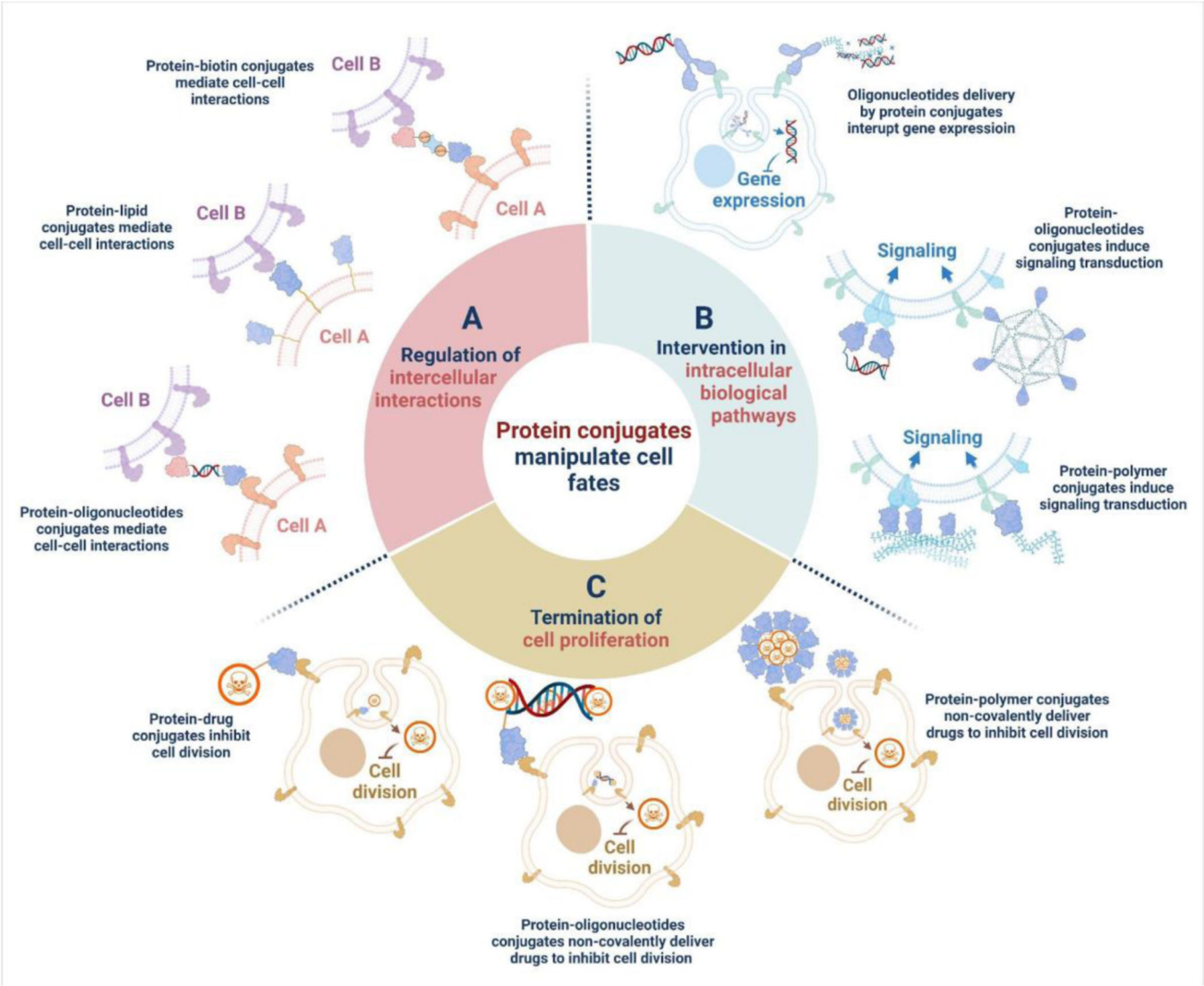

Figure 1. Manipulation of cell fates by protein conjugates.

Various types of protein conjugates have been developed as tools to manipulate cell fates by (**A**) regulation of intercellular interactions, (**B**) intervention in intracellular biological pathways, and (**C**) termination of cell proliferation. For each category, proteins were chemically conjugated with different functional molecules, including lipids, oligonucleotides, synthetic polymers, and other small molecules, for a wide range of biomedical applications.

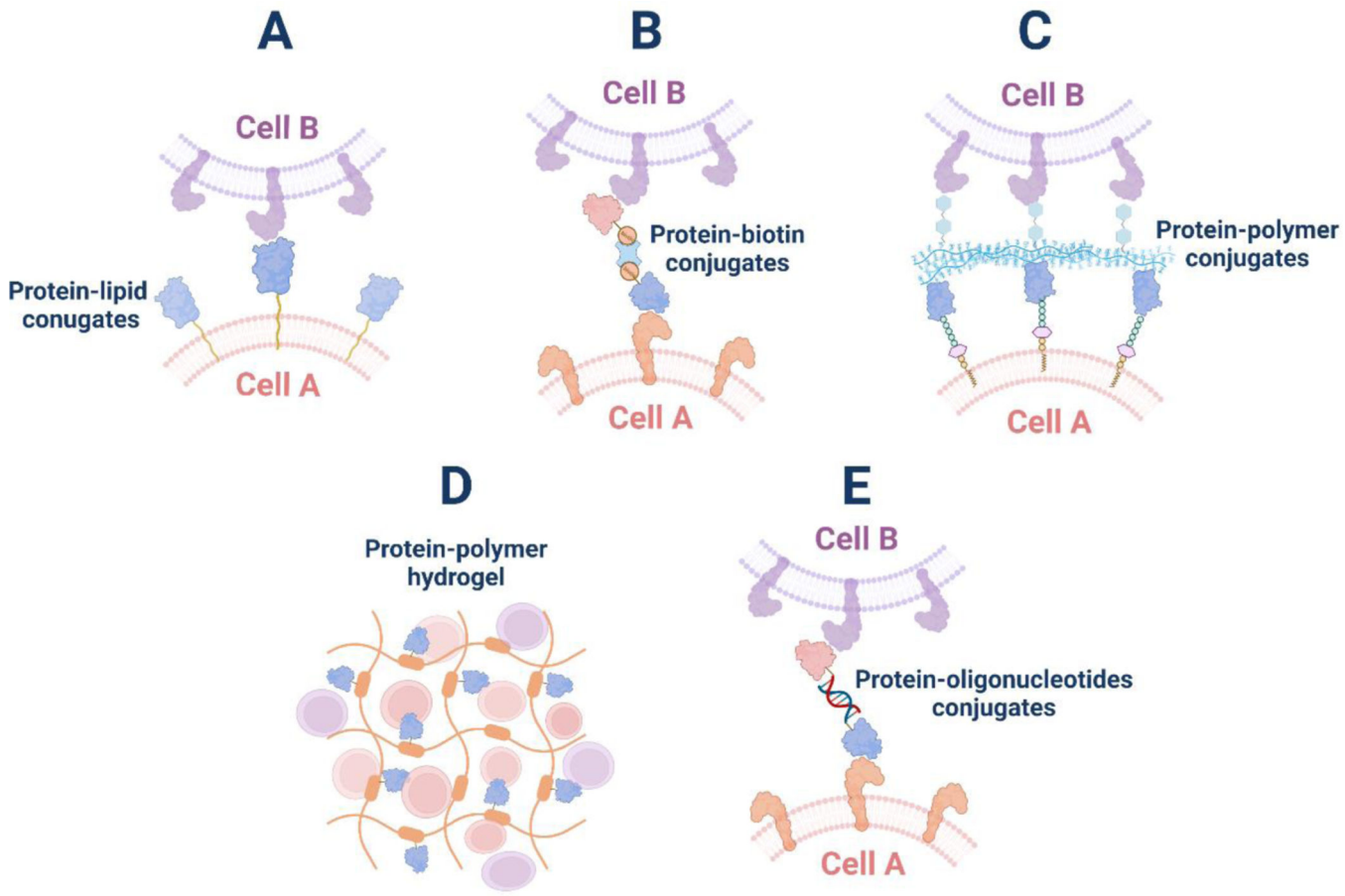

Figure 2. Different protein conjugates generated to regulate intercellular interactions.

(**A**) Protein-lipid conjugates have been used to modify cell surface and mediate cell-cell interactions. (**B**) Biotinylated proteins can induce cell-cell interactions through biotin-streptavidin bridging. (**C**) Protein-polysaccharide conjugates have been utilized to label the cell surface and mediate cell-cell interactions. (**D**) Synthetic polymers were modified with functional proteins to form hydrogels for the targeted delivery of cells to specific tissues and regulate their interactions. (**E**) Protein-oligonucleotides conjugates have been developed to program cell-cell interactions via oligonucleotides hybridization.

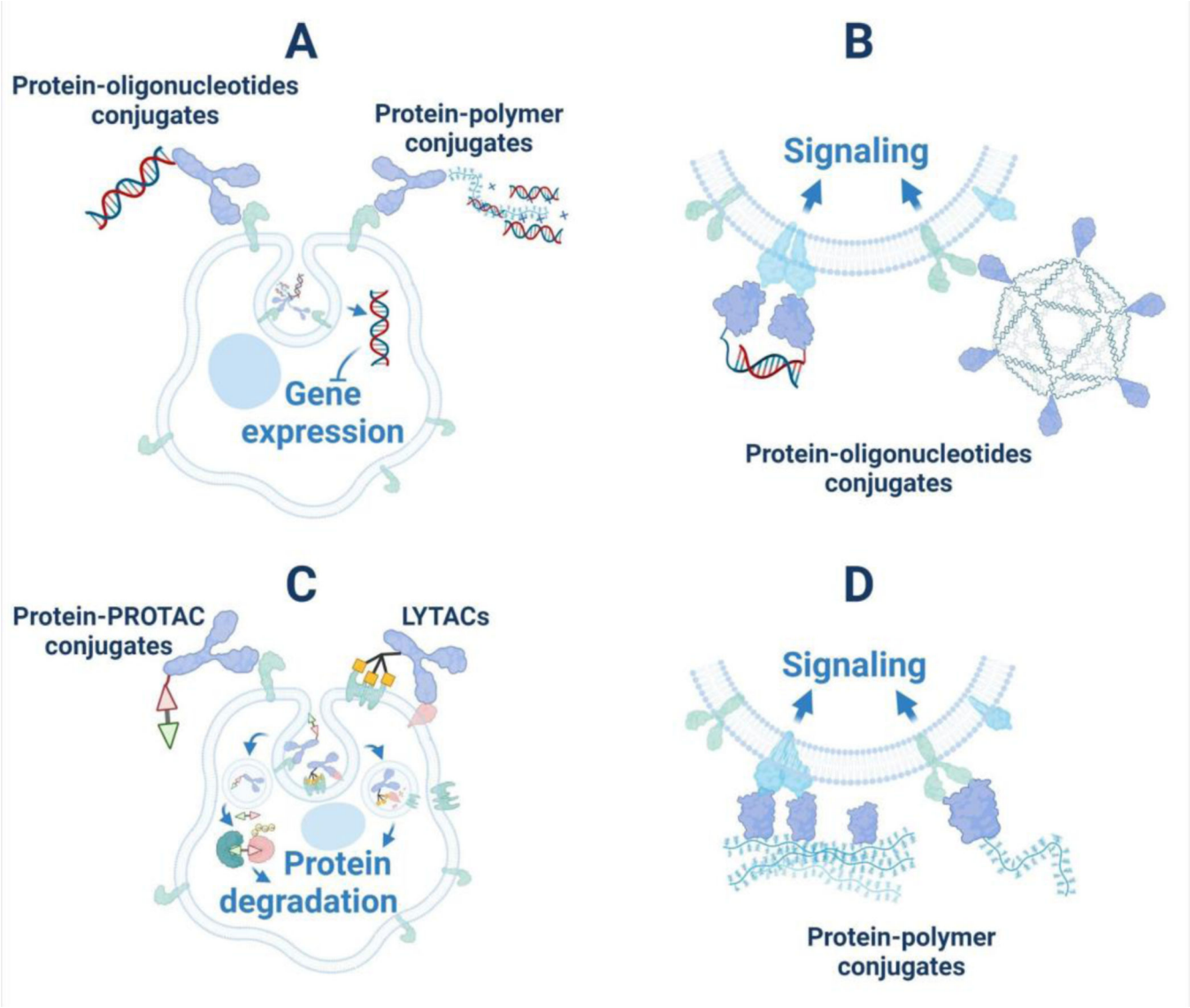

Figure 3. Different protein conjugates generated to intervene in intracellular biological pathways.

(**A**) Oligonucleotides covalently conjugated to proteins or non-covalently loaded onto positively charged protein-polymer conjugates for intracellular delivery to interrupt gene expression. (**B**) Protein-oligonucleotides synthesized to trigger cellular signaling by receptor binding and dimerization (or oligomerization). (**C**) Antibodies conjugated with small-molecule PROTACs or ligands for lysosome-targeting receptors to specifically degrade signaling proteins. (**D**) Functional proteins modified with synthetic polymers or conjugated to polymer-based hydrogels to activate receptor-mediated signaling of target cells or tissues.

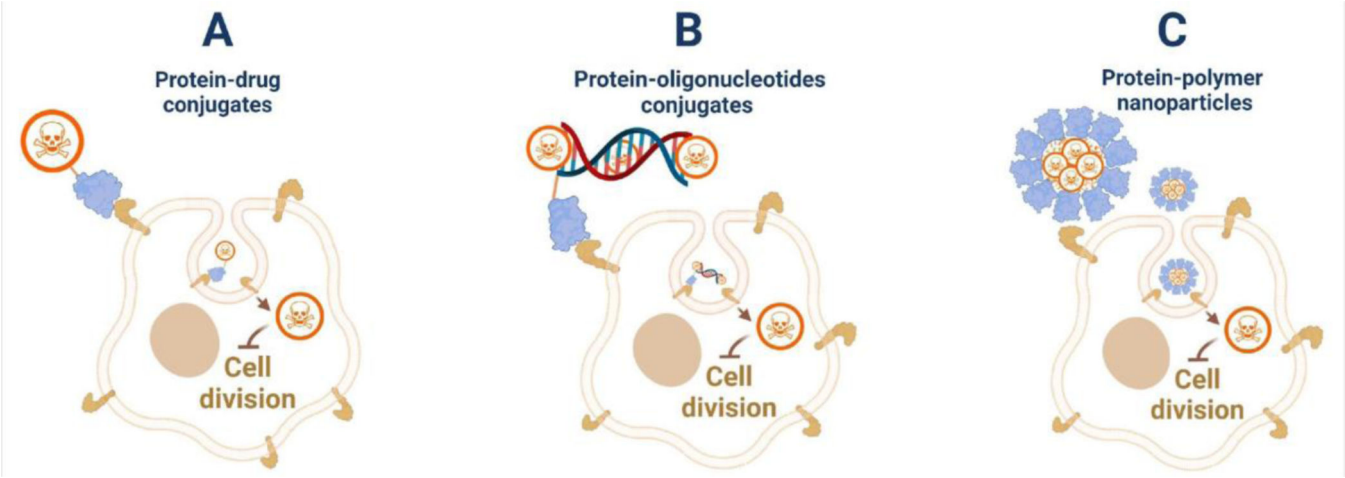

Figure 4. Different protein conjugates synthesized to terminate cell proliferation.

(**A**) Cytotoxic drugs have been conjugated to the antigen-specific proteins for targeted drug delivery. (**B**) Cytotoxic drugs can intercalate into oligonucleotides conjugated to targeting proteins for drug delivery. (**C**) Polymers can be coupled to proteins to form amphipathic conjugates that self-assemble into nanoparticles for the delivery of toxic drugs.

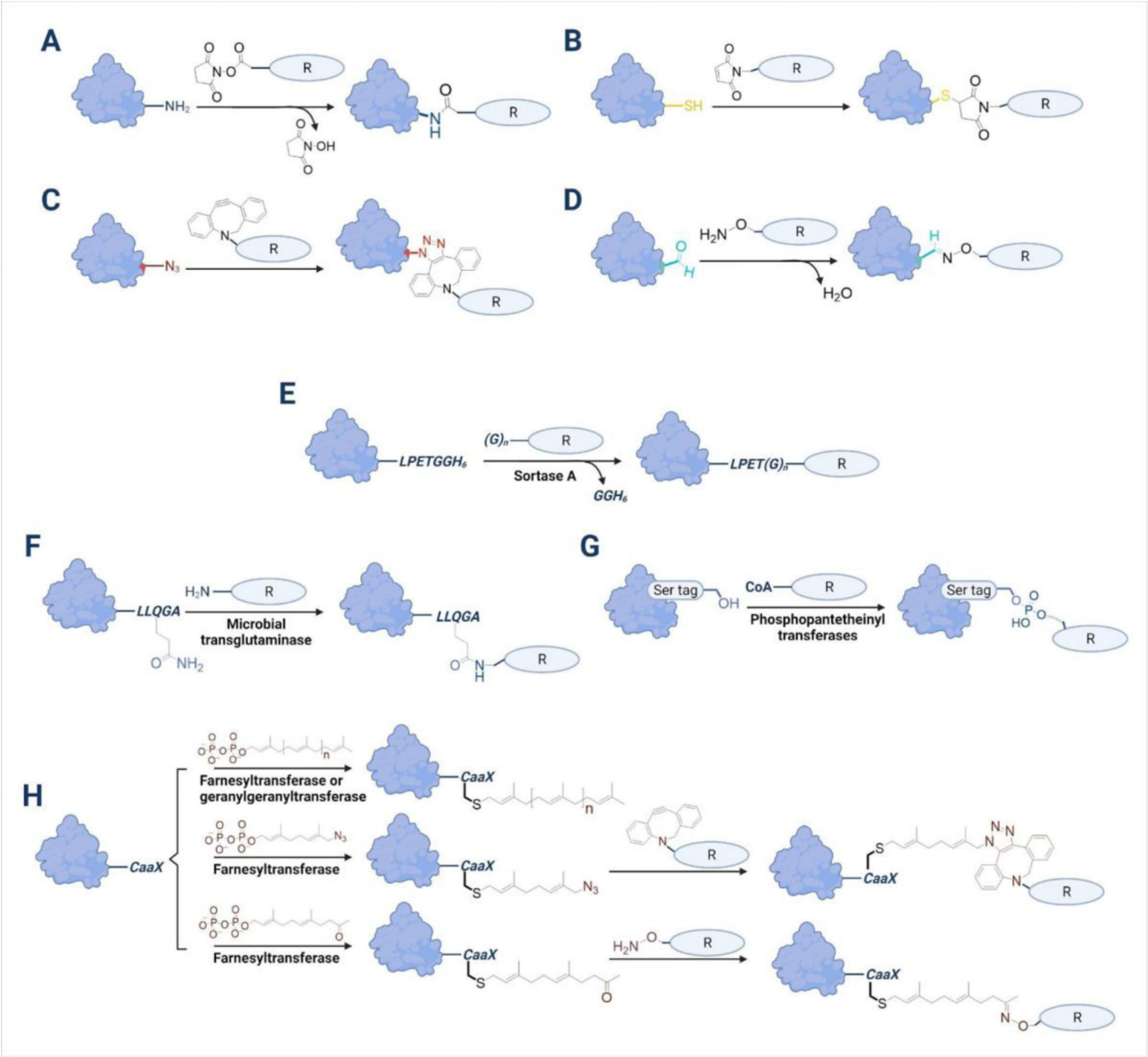

Figure 5. Different conjugation approaches to generate protein conjugates for the regulation of cell functions.

Most non-specific conjugations can be conducted by (**A**) reactions between primary amines and NHS esters or (**B**) reduced thiol coupling with maleimide-containing molecules. Unnatural amino acids were used to site-specifically incorporate (**C**) azide groups or (**D**) aldehyde groups into proteins for azide-alkyne cycloadditions or reactions with alkoxyamines respectively. Enzymatic reactions have also been employed as site-specific conjugation methods. (**E**) Sortase A was used to ligate functional peptides to proteins. (**F**) Microbial transglutaminase catalyzes the labeling of target proteins containing Q-tag sequences with lysine primary amine substrates. (**G**) Phosphopantetheinyl transferases modify serine residues with coenzyme A (CoA) derivatives. (**H**) Prenyltransferases

including farnesyltransferase or geranylgeranyltransferase can be used to modify proteins with natural isoprenoids or their derivatives functionalized with reactive groups for corresponding coupling reactions.