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Bioconjugate Therapeutics: Current Progress and Future Perspective

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Bioconjugate therapeutics refer to macromolecule drugs prepared through the attachment of therapeutic molecules to lipid or polymeric carrier molecules with covalent chemical linkers. A typical bioconjugate includes three basic building blocks: (1) carrier molecules such as polymers, lipids, peptides, proteins; (2) therapeutic agents including both small molecule chemicals and macromolecule drugs; and (3) chemical linkers. Since the therapeutic agents are covalently conjugated to carrier molecules, bioconjugate therapeutics are considered as macro-molecular prodrugs.

As a drug delivery strategy, the modification of therapeutic agents with carrier molecules have several advantages, including (1) optimal physical chemical properties, (2) enhanced disease specific targeting, (3) reduced toxicity, and (4) controlled drug release profile. One successful application of such strategy is PEGylation, which involves the conjugation of poly(ethylene glycol) (PEG) to therapeutic proteins. PEGylation of proteins can improve their stability and increase their plasma half-life. Several PEGylated protein products have been approved or in clinical development. Bioconjugates prepared with many other polymers, such as *N*-(2-hydrooxypropyl) methacrylamide (HMPA), have also demonstrated their potential in enhancing drug delivery and targeting. In these cases, hydrophilic polymers serve as the backbone for the attachment of drug molecules as well as other functional groups (e.g., targeting ligands, diagnostic agents). Usually, a multifunctional complex is prepared to not only increase drug solubility but also enhance drug targeting via both passive and active targeting. Although hydrophilic molecules are commonly used for bioconjugation, lipophilic molecules are also frequently used to enhance their cellular uptake and delivery to target organs and cells. Docosahexaenoic acid (DHA)–paclitaxel (PTX) conjugate is an example where DHA is conjugated to PTX to enhance tumor targeting. Similarly, hydrophobization and bioconjugation have also been proven to be an

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Special Issue: Bioconjugate Therapeutics

effective way to enhance the delivery and targeting of siRNAs. Because of the unique advantage of bioconjugation, it has been successfully used to enhance the delivery and targeting of various drugs including small molecules, proteins, peptides, and nucleic acids.

As the area of bioconjugate continues to develop, we have recently observed many innovations, new directions, and achievements. The development of material sciences and biotechnology provides a large spectrum of carrier molecules, which include polymers (both synthetic and natural polymers), peptides, and proteins (antibodies). New polymerization methods, such as reversible addition–fragmentation chain transfer polymerization (RAFT), offer the possibility to provide polymers with precisely controlled properties and narrow distribution of molecular weight. It will help to further reduce heterogeneity of conjugate therapeutics and thus address one critical challenge in product development. The design and synthesis of novel biodegradable and environment stimuli responsive materials will improve the safety and efficacy. The advances in recombinant DNA and protein engineering technologies have led to the progress in developing protein or peptide conjugated therapeutics. Last but not the least, antibody–drug conjugate is an emerging area with several antibody–drug conjugates being approved recently. In this theme issue, we included several review articles and research papers to highlight recent progresses in various aspects of research and development of bioconjugate therapeutics.

Synthetic polymer-based conjugates have been extensively used for the delivery of therapeutic and diagnostic agents. Since drug molecules are attached to the polymeric carriers through covalent linkers, the design and synthesis of polymer–drug conjugate is usually less flexible than physically encapsulated drugs in liposomes, nanoparticles, and micelles. The synthesis of polymer–drug conjugate is heavily dependent on the availability of functional groups and appropriate linkers. However, polymer–drug conjugates can provide high drug loading, prevent premature drug leakage, and release of drugs in a well-controlled manner. In addition, properly designed polymer–drug conjugates can target tumor and other disease lesions. In this theme issue, we included six papers belonging to this category. In the first paper, Kattel et al. investigated *in vivo* fate of polymer gemcitabine (P-GEM) conjugate. The conjugate was prepared by covalently attaching GEM with poly(ethylene glycol)-*block*-poly(2-methyl-2-carboxyl-propylene carbonate) bearing pendant carboxyl groups. The authors determined the short-term toxicity, pharmacokinetics, and biodistribution of free gemcitabine (F-GEM) and P-GEM after systemic administration in orthotopic pancreatic tumor bearing NSG mice. P-GEM improved the stability and sustained release of GEM, and enhanced drug delivery into the tumors. In another study, Karacivi et al. designed and synthesized a poly(oligoethylene glycol)methacrylate-based polymer–drug conjugate for bone targeted delivery of an antiangiogenic drug combretastatin A4 (CA4). This conjugate showed more cytotoxic against HUVECs and U2-OS cells than that in Saos-2. The third paper comes from Kopeček's group, who synthesized degradable HPMA copolymers conjugated with GEM or PTX through a RAFT copolymerization. The incorporation of GFLG peptide linker makes the polymer biodegradable. To investigate the effects of molecular weight, drug-conjugated polymers with different molecular weights, including diblock, tetrablock, and hexablock copolymer conjugates, were tested for their anticancer activities in nude mice bearing human ovarian xenografts. Among these tested conjugates, diblock polymer conjugates with a molecular weight of around 200 kDa showed

most potent anticancer activities as monotherapy for both PTX and GEM conjugates. In addition, sequential combination therapy using these conjugates further enhanced anticancer activities.

Polymer–drug conjugate can also be utilized for codelivery of drug and miRNA for combination therapy. Peng et al. investigated the use of HPMA-based polymer for codelivery of a CXCR4 antagonist, AMD3465, and a therapeutic miRNA, miR-200C mimic. The conjugate, named P-SS-AMD, was synthesized by conjugating AMD3465 to HPMA copolymer through a disulfide linker for effective intracellular drug release. Because of the cationic nature of AMD, P-SS-AMD was also able to function as a delivery carrier to mediate efficient transfection of miR-200C into cancer cells to inhibit the expression of its target gene ZEB-1. This system also showed enhanced inhibition of cancer cell migration.

The *in vivo* fate of polymers is critical for their clinical applications. The biodistribution and pharmacokinetics of polymers are greatly influenced by the design feature of polymers, such as molecular weight and degradability. Included in this theme issue are two research papers on the *in vivo* fate of HPMA copolymers. The first study was carried out by Fan et al. The accumulation of HPMA copolymers in mononuclear phagocyte system (MPS)-associated tissues is a concern for their clinical application as diagnostics and therapeutics. In this study, the authors synthesized multiblock HPMA copolymers with cathepsin S (Cat S)-cleavable peptide linkers. These HPMA copolymers can degrade in MPS-associated tissues, increase their clearance in these tissues, and thus reduce nontarget accumulation. These authors studied the effects of block size on the biological performance of their HPMA copolymers. The results indicated that HPMA copolymers with smaller block size (S-CMP) showed significant fast rate of cleavage than those with larger block size (L-CMP). *In vivo* biodistribution studies in mice bearing HPAC pancreatic ductal adenocarcinoma demonstrated that S-CMP had fast clearance and less retention in nontarget tissue while having good retention in the tumor. In the second paper, Wei et al. explored the application of I^{125}, Alexa Fluor 488, and IRDye 800CW-labeled HPMA copolymer–dexamethasone conjugates (P-Dex) as a theranostic agent for orthopedic implant loosening. P-Dex with larger molecular weight showed reduced elimination, decreased clearance, prolonged half-life, and increased systemic exposure. The accumulation of P-Dex to the peri-implant inflammatory lesion increased with the conjugate molecular weight.

Synthetic polymers have also been successfully used to deliver macromolecules such as protein, peptide, and aptamer. Chen et al. constructed a matrix metalloproteinase (MMP)-activatable cell-penetrating delivery system for delivering a plant protein toxin (Trichosanthin). First, a recombinant fusion protein containing trichosanthin, low molecular weight protamine, and MMP-2 substrate peptide was produced through genetic engineering. Then, maleimide-PEG was site-specifically conjugated to the recombinant protein with a C-terminal cysteine. Conjugated PEG can mask the nonspecific interaction of low molecular weight protamine, increase systemic half-life, reduce immunogenicity, and improve tumor targeting. This system can be cleaved by MMP upregulated in tumor microenvironment and unmask low molecular weight protamine to facilitate cellular uptake. The anticancer activity of this system was evaluated extensively with *in vitro* and *in vivo* studies. When used in combination with PTX, this conjugate system can overcome MDR through the reversal of

PTX-induced Caspase 9 phosphorylation and the induction of Caspase 3-dependent apoptosis. Similarly, Liu et al. designed a pH-responsive conjugate for targeted intracellular delivery of a protein toxin, RNase A. The authors conjugated RNase A to both PEG28-folate and PEG4-INF7 through an acidic cleavable traceless linker, AzMMMan. PEG-folate can enhance tumor targeting through folic acid receptor mediated cellular uptake. The INF7, an influenza hemagglutinin derived pH-sensitive peptide, can promote the endosomal escape. The use of AzMMMan linker can regenerate original RNase A and avoid negative impact on protein activity due to covalent modification. In addition, this system without cationic components may avoid issues related to cationic charge and further improve the biocompatibility. This delivery system demonstrated excellent anticancer effects on KB tumor cells *in vitro*. In the third study, Kern et al. designed a diblock polymer based system for intracellular delivery of BIM BH3 mimic peptide for cancer therapy. The first block is a pH-responsive endosomolytic copolymer composed of *N*,*N*′-diethylaminoeth-yl methacrylate (DEAEMA) and butyl methacrylate (BMA). At the physiological pH (7.4), this block promotes the assembly of polymeric micelles and functions as the hydrophobic core. At mild acidic pH, it can disrupt membranes and facilitate endosome escape. The second block was synthesized through copolymerization of polyethylene glycol methacrylate (PEGMA) and BIM BH3 mimic peptide conjugated meth-acrylamido-peptide macromonomer. BIM BH3 peptides were conjugated to the monomer through a four amino acid (FKFL) cathepsin B cleavable linker, which is cleaved by cathepsin B inside the target cancer cells and release BIM BH3 peptides. This study represents a good example of multicomponent smart carrier for intracellular delivery of peptides. Lee et al. reported the use of self-assembled conjugates for the delivery of apatmers. The authors prepared polymer–DNA nanoassembly with amplified aptamers having vascular endothelial growth factor (VEGF) capturing capacity for potential therapeutic applications in retinal vascular hyperpermeability and cancers. First, dextrans conjugated with complementary single-stranded DNA sequences were synthesized. These conjugates also contain VEGF DNA aptamers attached to the polymer backbone. Then, these conjugates form nanoconstructs through hybridization of two complementary single-stranded DNA sequences conjugated to dextran. VEGF DNA aptamers in the nanoconstruct were later amplified using a rolling circle amplification reaction to get the final product, polymer–DNA amplified aptamer nanoconstructs (PA-aNCs). PA-aNCs demonstrated great potential as anti-VEGF therapeutic agent. *In vivo* for inhibiting tumor growth and for treating VEGF induced retinal vascular hyperpermeability.

In addition to synthetic polymers, polypeptides and proteins have also been used as promising carrier molecules for bioconjugate therapeutics. Karuturi et al. constructed a bioconjugate vaccine (pp89-RR-EP67) composed of a host-derived mucosal immunostimulant (EP67), a protective MCMV CTL epitope (pp89), and a lysosomal protease-labile double arginine linker. pp89-RR-EP67 was encapsulated in PLGA nanoparticles and microparticles, respectively. The results indicate that the particle size plays a significant role in determining the magnitude, memory subsets, and epitope-specific CD8+ T cells of this vaccine. The study, by Rad-Malekshahi et al., developed self-assembling peptide epitopes (SAPEs) as vaccine delivery systems by conjugating a self-assembling peptide or a thermosensitive polymer to the N-terminus of peptide agents. SAPEs form

nanoparticles and effectively induce and expand antigen specific CD8+ T cells in mice when used in combination with CpG. Treatment with SAPEs containing HPV E743–57 peptide delayed tumor growth and prolonged the survival of tumor bearing mice. As a vaccine delivery system for tumor immunotherapy, SAPEs have several advantages: (1) reducing premature antigenic epitope release and thus minimizing T cell anergy, and (2) enhancing cellular uptake of vaccine and immune response. In another study, Zhao et al. fused single-chain variable fragment (scFv) of the anti-PD-1 antibody with elastin-like polypeptides (iTEPs). The fusion protein forms nanoparticles and works as effective as full-sized anti-PD-1 antibody in blocking the PD-1 immune checkpoint *in vitro* and *in vivo*. The study presented multivalent aPD-1 nanoparticles, which can diversify the group of the PD-1 antibodies and provides a platform to build targeted delivery systems for the antibody.

Antibody–drug conjugate (ADC) is a class of therapeutic agents that are prepared through conjugating drugs to specific antibodies. Currently, there are two FDA-approved ADCs (i.e., Kadcyla and Adcetris) and more than 40 ADCs in clinical trials. This study, by Dimasi et al., presented a method to prepare ADCs through site-specific conjugation. Cysteines are inserted adjacent to the selected sites in the antibody and are available to form conjugations with a maleimide-bearing drug (SG3249). ADCs prepared with this method are more homogeneous, which is critical for their clinical applications. A selected ADC with cysteine-insertion after position 239 demonstrated potent anticancer activity in mice bearing xenograft breast tumors.

Lipid–drug conjugate provides an alternative strategy to enhance the delivery of various drugs including small molecule chemicals, peptides, proteins, and siRNAs. The conjugation of lipids molecules can increase lipophilicity and alter other properties. Lipid–drug conjugates have been used to improve oral bioavailability, achieve lymphatic targeting, and enhance tumor targeting. Li et al. have extensively reviewed the design and application of lipid–drug conjugates for enhancing drug delivery. The authors introduced different lipids, chemical linkers, and conjugation strategies used for preparing conjugates. The advantages of lipid–drug conjugates were highlighted with numerous application examples. In addition, various delivery carriers used to formulate lipid–drug conjugate, such as emulsions, liposomes, micelles, lipid nanoparticles, and polymer nanoparticles, were summarized. The lipid moieties in the conjugates can facilitate their encapsulation in delivery carriers and generate formulations with high drug loading capacity and excellent stability.

Small interference RNA (siRNA) has attracted great attention as a therapeutic agent for various diseases. However, the *in vivo* delivery of siRNA to target tissues or cells remains a challenge. The review by Baumer et al. summarized recent efforts for improving the *in vivo* delivery of siRNA. The authors compared various approaches to stabilize siNRA in different bioconjugates. In addition, they also discussed the use of different targeting ligands including antibody, scFv, and Fab fragment for targeting specific siRNA delivery. In another paper, Jain et al. optimized the multicomponent siRNA–streptavidin–cholesterol–protamine (SSCP) nanocomplex for efficient siRNA delivery. This system includes biotin-conjugated siRNA with a cleavable disulfide linker, biotin-conjugated cholesterol as a targeting ligand for hepatic stellate cells, avidin analogues to facilitate the forming of nanocomplex, and positively charged protamine as a condensing agent. In this study, they compared avidin-,

neutravidin-, and streptavidin-based systems and found that the neutravidin-based system showed best performance in delivering siRNA into hepatic stellate cells. It is due to low endosome entrapment and high cytosolic localization of neutravidin-based system. In addition, neutravidin-based system showed no immunogenicity and efficient accumulation in liver of rat with liver fibrosis.

Conjugation chemistry has also been used for the modification of particulate drug delivery systems. We included two papers in this theme issue where the surfaces if liposomes and nanoparticles were modified through conjugation chemistry to improve their drug delivery performance. Sialyl LewisX (sLeX) is a promising ligand for targeting inflamed and tumor endothelium overexpressing E-selectin. However, its application is limited due to the complicated synthesis method, which involves multiple reaction steps. Chantarasrivong et al. synthesized novel sLeX analogues with simplified structures and conjugated it to 1,2-distearoyl-sn-glycero-3-phosphoetha-nolamine-polyethylene glycol-2000 (DSPE-PEG). sLeX analogues conjugated DSPE-PEG was incorporated in the formulation to prepare E-selectin targeting liposomes. The liposomes showed high uptake by human umbilical vein endothelial cells (HUVECs) treated with inflammatory cytokines and thus demonstrated its potential for targeting tumor tissues or endothelium of inflamed tissues. The study, by Han et al., provided a method to generate functional nanoparticles, which can target tumors by utilizing acidic tumor microenvironment. Authors conjugated PLGA nano-particles (NPs) with amidated TAT peptide. The modified TAT peptide showed pH-dependent protonation and depro-tonation. NPs decorated with this peptide showed minimal interaction with cells at pH 7.4 while showing enhanced interaction with cells at pH 6.5. However, the pH-sensitivity observed from *in vitro* studies was lost during *in vivo* studies, possibly due to the difference in the activation and opsonizing of NPs by murine blood and bovine serum containing medium.

The conjugation with cellular components represents a less visited but evolving research area. Included in this theme issue are two papers belonging to this category. In the first paper, Xia et al. described a novel approach to inhibit Grb2-Sos1 protein interaction by site-specific conjugation of reactive peptide with the targeting protein. In this study, a reactive peptide was designed to specifically bind to $Grb2^{N\text{-}SH3}$ and form an irreversible covalent bond. The conjugation of peptide to Grb2 nearby the Sos1 binding site can effectively block Grb2-Sos1 interaction. The authors demonstrated that optimized reactive peptide could conjugate with endogenous Grb2 protein inside SK-BR-3 human breast cancer cells and reduced cell mobility and viability. This study showed the possibility of designing site-specific covalent inhibitors and their potentials for cancer treatment. In another study, Kwon et al. showed the application of metabolic engineering and bioorthogonal click reaction as an alternative active tumor targeting approach to enhance the tumor targeting of nanoparticles. The heterogeneous nature of tumors leads to a significant challenge in the design of active tumor targeting nanoparticles with biological ligands. To address this issue, the authors labeled tumors with surface azide functional groups by treating cells with *N*-azidoacetylmannosamine-tetraacylated (Ac4ManNAz). In the presence of Ac4ManNAz, unnatural glycans containing azide group was generated through glycan biosynthetic pathway. Bicyclononyne (BCN)-conjugated glycol chitosan nanopar-ticles (BCN-CNP) were used as azide-reporter-targeting NPs. Cyclic RGD-conjugated glycol chitosan nanoparticles

(cRGD-CNP) was also synthesized as a control. BCN-CNP demonstrated higher cellular uptake than cRGD-CNP. The former also showed a relatively uniform distribution. This study provided an alternative approach for active tumor targeting.

We also included in this issue a comprehensive review on nanoparticle-based contrast agents for magnetic resonance imaging (MRI). In this review, the authors induced various methods of synthesizing magnetic iron oxide nanoparticles (MIONs) with focus on liquid-based synthesis approaches. In addition, the application of MIONs as MRI contrast agents including negative contrast agents (MIONs larger than 10 nm) and positive contrast agents (extremely small MIONs smaller than 5 nm) were summarized.

In summary, we would like to thank all the authors for their contributions to this theme issue of *Molecular Pharmaceutics*. This issue highlighted recent status, achievements, and challenges in bioconjugate therapeutics, which are not only scientifically interesting but also have great potential for commercialization. However, in contrast to the huge number of publications, the product development and clinical applications remain fairly low. Bioconjugates as multicompo-nent complex systems are significantly different from those simple small molecule drugs. As new chemical entities (NCE), they still need to satisfy the stringent requirements for regulatory approval by the FDA. Therefore, the product development of bioconjugate therapeutics has to address some additional challenges in identifying critical product attributes, developing validation methods, and controlling product quality. We hope this issue could encourage the talk between bench-side scientists and those who are involved in the product development and commercialization. This type of communication will increase the awareness and understanding of critical challenges in innovation and product development.

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