MINI-REVIEW

Bioresponsive nanogels for protein delivery

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Abstract
Protein drugs have attracted more attention due to their high specificity and efficacy. However, the poor stability, plasma degradation, inferior cell membrane permeability, and immunogenicity severely limit the in vivo application of protein drugs. Nanogels are the nanosized crosslinked gels with high water-loading capacities and large cavities for protein loading, which are able to increase the stability and decrease the immunogenicity for protein delivery. The bioresponsive nanogels possess the capability of programatically releasing the protein drugs in an on-demand manner at the target sites with distinct biosignals, which show considerable potential to increase the therapeutic efficacies and decrease the adverse effects of the protein drugs. In this review, we outline the recent advance in the bioresponsive nanogels for delivery of protein drugs, and survey the design of new materials and formulations that can respond typical biosignals, such as temperature, pH, reductive potential, and enzyme expression. The prospects and challenges are also discussed.

KEYWORDS
biosignals, controlled release, drug delivery, nanogel, protein drugs

1 | INTRODUCTION

Since the first biotech drug, human recombinant insulin came onto the market in 1982, the protein drug is experiencing rapid development and showing great potential for treatment of diseases. During the recent 5 years, the protein drugs approved by the U.S. Food and Drug Administration (FDA) account for approximately a third of total approved drugs. These protein drugs are widely used in clinic for the treatment of cancer, metabolic diseases, and infectious diseases. Protein drugs possess high activity and specificity with reduced toxicity in comparison with small-molecule chemical drugs. However, most of them suffer from instability, plasma proteolysis, poor transmembrane ability, and immunogenicity.

Considerable efforts have been made to enhance the stability of protein drugs, such as modification of protein drugs with biocompatible polymers. Polyethylene glycol
TABLE 1  Summary of nanogels with different responsibilities discussed in this review

| Biosignals | Stimuli | Response moieties | Protein drugs | Application | Reference |
|------------|---------|-------------------|---------------|-------------|-----------|
| Temperature | > 32 °C | PNVCL | OVA | Transdermal immunization | 34 |
|            | > 32–49 °C | P(NIPAM-co-NCPAM) | BSA | N/A | 36 |
| pH         | < pH 6.8 | Protonable carboxyl group | RWrNR-kla | Cancer | 40 |
|            | < pH 6.5–6.8 | Protonable carboxyl group and amino group | IL-2 | Cancer | 41 |
|            | < pH 5.0 | Hydrazine bond | OVA | Cancer | 44 |
|            | < pH 6.5–6.8 | Bis-substituted maleic anhydride-amine | Cyt C | Cancer | 46 |
|            | < pH 5.0–6.0 | Mono-substituted maleic anhydride-amine | | | |
|            | < pH 5.0 | Citraconic amide | DNase I | Cancer | 47 |
| Reduction  | GSH | Disulfide bond | Cyt C | Cancer | 53 |
|            | GSH | Disulfide bond | Synthetic long peptides | Cancer | 55 |
|            | GSH/DTT | Disulfide bond | Caspase-3 | Cancer | 57 |
|            | GSH | Disulfide bond | IL-15Sa | T cell activation | 59 |
|            | GSH | Disulfide bond | IL-2/Fc | Alloimmunity | 61 |
|            | GSH | Disulfide bond | S-RBD | Coronavirus | 62 |
| Enzyme     | Plasmin | GGKFKTGG | Bone morphogenetic protein-2 | Osteogenesis | 66 |
|            | MMP-2 | GALGLP | PON-1 | Atherosclerosis | 68 |
|            | Thrombin | LVPRGS | Hirudin | Thrombosis | 69 |

Abbreviation: N/A, not applicable.

(PEG) modification is a favorable approach to prolong the half-life of protein drugs and reduce the unexpected degradation in blood circulation. Several PEGylated protein drugs have been approved by the FDA for the market, such as the PEGylated L-asparaginase for the treatment of acute lymphoblastic leukemia and the PEGylated antitumor necrosis factor-α antibody for the treatment of rheumatoid arthritis. However, there are still existing problems in the chemical modification strategy that may cause partial loss of protein bioactivity due to modification in the structure of the amino acid at or near the active site. The application of nanocarrier, including liposome, polymeric micelle, and inorganic nanoparticle, is an alternative solution to increase the stability of protein drugs, protect them against enzymatic degradation, promote intracellular delivery, and reduce immunogenicity.

Nanogels are swollen crosslinked gels with nanoscale size and high water-loading capacity. The water-containing cavity is environmentally beneficial to the entrapment and delivery of proteins. The crosslinked network can restrict the free movement of the encapsulated proteins to a certain extent, thereby decreasing aggregation and increasing stability. Moreover, the nanogels not only provide a protective shell for protein drugs against proteases, but also enhance the transmembrane and intracellular delivery of protein drugs. The constituent materials of nanogels are mainly natural polysaccharides, such as pullulan, hyaluronic acid, chitosan, dextran, and synthetic polymers, such as polyacrylamide. Functionalization with targeting ligands allows the nanogels to...
more accumulate to the lesion tissues or cells. More importantly, incorporation of bioresponsive materials endows the nanogels with programmable properties in response to the physiological or pathological signals, which can overcome key challenges associated with conventional nanosystems, and enhance the protein delivery efficiency and therapeutic efficacy. In this review, we summarized recent advances in the bioresponsive nanogels for protein delivery (Figure 1). Representative nanogels in response to typical biosignals with the responsive moieties, protein drugs, and biomedical applications are listed in Table 1. Perspectives and challenges are also discussed.

2 \ THERMORESPONSIVE NANOGELS

Thermoresponsive nanogels are composed of thermosensitive polymers, such as poly(N-isopropylacrylamide) (PNIPAM), poly(N-vinylcaprolactam) (PNVCL), poly(N-cyclopropylacrylamide) (PNCPAM), and poly (L-lactide) (PLA). Upper critical solution temperature (UCST) and lower critical solution temperature (LCST) are two main parameters of the thermosensitive polymers. When the environmental temperature is higher than UCST or lower than LCST, the mixture was homogeneous, otherwise incompatible. For example, PNIPAM is soluble in the aqueous solution at the temperature below 32 °C that is its LCST, but to be insoluble with precipitation above 32 °C. The nanogels consisting of thermosensitive polymers can release the encapsulated protein in response to the surrounding temperature. The solubility of polymers changes with the environmental temperature varying across their LCSTs or UCSTs, leading to the volume expansion or shrinkage of nanogels and subsequent release of the loaded proteins. In the human body, the temperature variation often occurs under pathological conditions, such as inflammatory or tumor tissues, which can be used to modulate the protein release at the target tissues.

PNVCL is a biocompatible and thermosensitive polymer with a transition temperature of 32 °C that is close to the skin surface temperature. Sonzogni et al. developed PNVCL-based nanogels and evaluated their protein delivery ability for transdermal immunization. The average diameter of the obtained PNVCL nanogels reduced from 257.8 to 157.9 nm with the increase of temperature from 25 to 37 °C, indicating that the nanogels possessed thermoresponsiveness with the transition temperature determined to be 31.4 °C. Ovalbumin (OVA), a model antigen, was efficiently loaded in the PNVCL nanogels with the encapsulation efficiency up to 94%, and was responsively released from the nanogels upon the temperature increase. The PNVCL nanogels could improve the penetration of OVA in the barrier-deficient skin, which is beneficial to transdermal immunization.

The LCST can be tuned by adjusting the polymer component. The LCSTs of PNIPAM and PNCPAM are 32 and 49 °C, respectively. P(NIPAM-co-NCPAM) obtained by copolymerization of NIPAM and NCPAM had an LCST ranging from 32 to 49 °C with the varied ratio of NIPAM and NCPAM. Massi et al. synthesized P(NIPAM-co-NCPAM) with different monomer ratios and examined their physical properties. The higher molar ratio of NIPAM and NCPAM, the higher phase transition temperature. Further modification of P(NIPAM-co-NCPAM) with PEG could form thermosensitive polymer PEG-P(P(NIPAM-co-NCPAM), which changed from hydrophilic into amphipathic when the temperature rose over LCST. This process was favorable for assembly of nanogels with simultaneous encapsulation protein drugs.

3 \ pH-RESPONSIVE NANOGELS

Variation of pH exists in different tissues and organelles. For example, the tumor microenvironment is mildly acidic (pH 6.5-6.8) because of the increased lactic acid levels caused by excessive glycolysis. In the cells, the pH values of the endosomal, lysosomal, and cytoplasmic internal environments are 5.0-6.0, 4.0-5.0, and 7.4-8.0, respectively. These pH differences have been widely used to develop pH-responsive nanogels for site-specific release of antitumor proteins for cancer treatment.

The first potential strategy for designing pH-responsive nanogels is introduction of ionizable moieties into the polymer components, such as ionizable amino or carboxyl groups. Isoelectric point (pI) is a characteristic constant of ionizable polymers, which defines the net charge of the polymer is zero as the solution pH is equal to pI. When the pH > pI, the polymer has a negative charge, and on the contrary, a positive charge. Xu et al. prepared a pH-sensitive nanogel that was assembled by electrostatic interaction. A positively charged apoptotic peptide (kla) was chemically conjugated with RWrNR that is a targeting ligand to the integrin αvβ3 receptor to form a cationic peptide RWrNR-kla. Poly(L-arginine) (Parg) was also a cationic peptide, and could transfer into nitric oxide (NO) by nitric oxide synthase after internalization by the tumor-associated macrophages. Both kla and NO are toxic to the tumor cells. To realize a synergistic anti-tumor effect of RWrNR-kla and Parg, negatively charged carboxymethyl chitosan (CMCS) with the pI value of 6.8 was incorporated with RWrNR-kla and Parg to assemble pH-responsive nanogels by electrostatic interaction under neutral conditions. In the tumor microenvironment (pH < 6.8), the CMCS in nanogels transferred to be positively charged, leading to the collapse of nanogels and release of RWrNR-kla and Parg due to electrostatic repulsion. The
ligand-receptor binding effect between RWrNR and the integrin \(\alpha_v\beta_3\) promoted the penetration of RWrNR-kla into the tumor cells, leading to efficient induction of mitochondrial-mediated apoptosis. On the other hand, the uptake of Parg by the tumor-associated macrophages elevated the level of NO, which could freely enter into the tumor cells and induce the production of reactive oxygen species (ROS). Increased intracellular level of ROS eventually induced tumor cell death. The combination of these two pathways significantly enhanced the antitumor effects than monotherapy, which efficiently inhibited the growth of the murine melanoma (B16F10) xenograft tumors in the mouse model. Song et al. developed a biomimetic pH-responsive nanogel with a core-shell structure for delivery of interleukin (IL)-2, which was constructed by red blood cell (RBC) membrane-coated with a pH-sensitive nanogel core. The nanogel core was composed of cationic methacrylamide N-(2-hydroxy)propyl-3-trimethylammonium chitosan chloride (HTCCm) and amphiphilic N-carboxyethyl chitosan (CECm). Its pH-sensitivity was modulated by changing the ratio of HTCCm and CECm. The RBC membrane envelop provided the nanogel with a stealth ability and prolonged circulation time in the blood. Meanwhile, the proteins on the RBC membrane had the homogenous structure of IL-2, which assisted the adsorption and delivery of IL-2. In the acidic tumor microenvironment, the nanogel swelled rapidly, leading to the release of the encapsulated paclitaxel, a chemotherapeutic drug and the disintegration of the RBC membrane, accompanied with the release of IL-2. This pH-triggered site-specific release of paclitaxel and IL-2 achieved a synergistic effect of chemotherapy and immunotherapy. The nanogel showed strong effect on stimulating the antitumor immunity and suppressing the tumor growth in the B16-F10 tumor-bearing mice. Incorporation of pH-degradable chemical linkages, such as hydrazone, acetal, thioketal, and ester groups in the polymer components, is an alternative approach to developing pH-responsive nanogels for protein delivery. Wang et al. synthesized an acid-sensitive amphiphilic dextran derivative, which consisted of all-\textit{trans} retinal-conjugated dextran via a hydrazone bond. All-\textit{trans} retinal is favorable for maturation of dendritic cells, which bound with the retinoic X receptor or retinoic acid receptor to exert immunoregulatory effect of the dendritic cells. Further modification of galactose to dextran rendered active targeting to the dendritic cells with the C-type lectin receptor. This amphiphilic galactosyl dextran-retinal (GDR) polymer could self-assemble into nanogels and encapsulate OVA. After internalized by the dendritic cells, the nanogels degraded in the acidic endocytotic vesicles through the cleavage of the hydrazone bond, and subsequently released all-\textit{trans} retinal and OVA to promote the maturation of the dendritic cells, which markedly enhanced the proteasome activity the antigen presenting of the dendritic cells. The in vivo effect of GDR/OVA on tumor growth inhibition was verified in the xenografted melanoma (B16-OVA) mouse models. Hu et al. had prepared a pH-responsive protein drug depot for antitumor therapy. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and cilengitide were coloaded in a pH-responsive nanogel that was constructed by neutral monomer and an acid-cleavable crosslinker, glycerol dimethacrylate using emulsion-based polymerization. This pH-responsive nanogel was further modified with human serum albumin (HSA) and transglutaminase (TG)-loaded hyaluronic acid (HA) nanogel to form CS-NG. After systemic administration, CS-NG accumulated in tumor microenvironment. The overexpressed hyaluronidase (HAase) triggered the degradation of the HA nanogel and the release of cytokine. TG catalyzes the crosslinking of HSA and surrounding proteins, leading to the formation of drug depot. The mild acid microenvironment continuously caused the degradation of depots and the release of antitumor protein drugs, TRAIL and cilengitide for combination anticancer effect. Recently, Su et al. developed a dual pH-responsive nanogel based on a reversible acid-sensitive linker, maleic anhydride with adjustable degradation conditions. Mono-substituted maleic anhydride degrades under mildly acidic environment (pH 6.5-6.8), while bis-substituted degrades under acidic environment (pH 5.0-6.0). Cytochrome C (Cyt C) is an apoptotic protein, which can induce the apoptosis of tumor cells via the caspase pathway. Transportan 10 (TP10) is a cell-penetrating peptide that can enhance the intracellular accumulation of the nanogels. Both Cyt C and TP10 are lysine-rich substances with free amino groups that can reversely react with maleic anhydride. 4-arm-PEG was used as a crosslinker to prepare the nanogels. The end of each arm was monosubstituted maleic anhydride, which crosslinked with Cyt C and TP10 to form positive charged nanogel MA-NG. The unreacted amino groups on Cyt C or TP10 were further conjugated with carboxy-dimethylmaleic anhydride (CDM), a bis-substituted maleic anhydride to obtain anionic nanogel CDM-MA-NG. After the nanogels reached the mildly acidic tumor stroma, the bis-substituted maleic anhydride derivate degraded, leading to the left of CDM from CDM-MA-NG and charge reversal of nanogels. Cationic MA-NG could be internalized into tumor cells with the help of TP10. In the lysosomes, the mono-substituted maleic anhydride derivate degraded, resulted in the traceless release of Cyt C and TP10 into the cytoplasm. This dual pH response nanogel significantly reduced the tumor size and prolonged the survival rate of HeLa tumor-bearing mice.
Our group proposed a pH-activated self-degradation strategy for nanogel-shuttled protein delivery. HA modified with cholesteryl and methacrylic acid ester (cm-HA) was synthesized. cm-HA could self-assemble into the nanogels in the aqueous solution by the hydrophobic interactions of the cholesterol moieties, and be highly stabilized by the free radical-mediated chemical crosslinking of methacrylic groups. The obtained nanogels showed high loading capacity for proteins with different pI and molecular weights, such as deoxyribonuclease I (DNase I), ribonuclease A (RNase A), and bovine serum albumin. To enhance the protein delivery efficiency, an acid-activated hyaluronidase (aHAase) was encapsulated in the nanogels, which was engineered by shielding the free amino residues of the native HAase with the citraconic anhydride modification. After intravenous administration, this physical and chemical collaborative assembly approach provided the nanogel with prolonged circulation and low leakage of DNase I. Once the nanogel accumulated in the tumor tissue, the mild acidity of the tumor microenvironment caused partial activation of aHAase due to acid-triggered citraconic amide degradation, followed by swelling of the nanogel and release of aHAase. The reactivated aHAase could degrade HA, a main component of the extracellular matrix of the tumor tissue for increased tumor penetration of the nanogel. Subsequently, aHAase was fully activated in the endocytotic vesicles with lower pH after the nanogel was taken up by the tumor cells, which completely degraded the HA-based nanogel and intracellularly released DNase I to cause tumor cell death by digesting DNA. The acid-triggered self-degradable nanogel showed high DNase I delivery and antitumor efficiencies on the human lung tumor (A549) xenografted mouse model. Furthermore, we developed a hierarchically assembled nanogel to overcome multiple physiological barriers for delivery of RNase A and to improve antitumor efficacy by eliminating cancer stem-like cells (CSCs) in combination with doxycycline, an anti-CSC agent. RNase A was encapsulated in a redox-responsive single-protein nanocapsule (R-rNC) with a small size (~8 nm), which was coloaded with doxycycline in an acid-degradable polymeric nanogel with a large size (~100 nm). The tumor acidity caused the degradation of the nanogel, accompanied by simultaneous release of R-rNC and doxycycline. Owing to decreased diffusive resistance, the small-sized R-rNC and small-molecule doxycycline could efficiently penetrate the tumor tissue. RNase I could be released from R-rNC within the reductive cells and degrade the cellular RNA leading to the tumor cell death, while doxycycline suppressed mitochondrial biogenesis to kill CSCs. Treatment with this hierarchically assembled nanogel resulted in high antitumor efficacy on the CSC-enriched breast tumor (MDA-MB-231) xenografted mouse model.

4 REDUCTION-RESPONSIVE NANOGELS

Glutathione acts as an essential regulator of cellular redox states in mammalian cells, and exists mainly in its reduced form, γ-glutamylcysteinyl glycine tripeptide (GSH). GSH together with GSSH, the oxidized form of glutathione maintains and regulates the cellular redox status, which is related to a variety of physiological functions, including xenobiotic detoxification, amino acid transportation, and protein synthesis. The intracellular concentration of GSH (0.5-10 mM) is much higher than extracellular (2-20 μM). Moreover, the intracellular concentration of GSH within the tumor cells was also higher than that in the normal cells. These concentration variations have been applied as signal triggers to design redox-responsive nanoparticles for drug delivery. 50–52 Li et al. developed a GSH-labile nanogel with intrinsic fluorescence for delivery of Cyt C. Hydrophobic moiety tetrazole-oligo (ethylene glycol) was first grafted to HA to form an amphiphilic polysaccharide HA-OEG-Tet. Subsequently, a disulfide bond-containing linker, dimethacrylamide-modified L-cystine (MA-Cys-MA), was synthesized. The redox-responsive nanogel was prepared by HA-OEG-Tet and MA-Cys-MA through an inverse nanoprecipitation method upon UV irradiation. The UV irradiation triggered the crosslinking of tetrazole and alkene to form pyrazoline group, which stabilized the nanogel. During this process, Cyt C could be encapsulated in the nanogel. Interestingly, this nanogel containing the pyrazoline groups exhibited a strong fluorescence emission at 510 nm, which is favorable for observing its intracellular behavior. The in vitro studies demonstrated the GSH-triggered release of Cyt C and good targetability of nanogel to the CD44-positive human breast cancer (MCF-7) cells. The in vivo studies proved that the nanogel significantly inhibited tumor growth in the MCF-7 tumor inoculated mice.

Apart from the tumor cells, several immune cells, including the dendritic cells, express high level of GSH. Kordalivand et al. prepared a redox-sensitive dextran-based nanogel for peptide-mediated cancer immunotherapy. The methacrylated molecules, including cationic monomer and pyridyl disulfide-containing monomer, were synthesized, which were reacted with methacrylated dextran in a water-in-oil inverse emulsion system to form the nanogels. The pyridyl disulfide groups were further reacted with the amino groups of synthetic long peptides that could promote maturation of the dendritic cells and evoke antitumor immunity. The nanogel could be internalized into the dendritic cells and release the synthetic long peptides under reductive circumstance in the cytosol. The released peptide containing epitopes effectively activated the cytotoxic
T cells and CD4+ T helper cells for enhanced cancer immunotherapy.

Free radical-mediated polymerization of vinyl monomers in the inverse emulsion system has been widely used for the preparation of the nanogels, in which the vinyl groups may also react with the thiol groups on cysteine residues of proteins, leading to the inactivation of proteins. To solve this issue, Raghupathi et al. modified caspase-3 with cysteine to shield its activity. In the presence of reduction substances, such as GSH or dithiothreitol (DTT), disulfide bond degraded and caspase-3 recovered its intact structure. This engineered caspase-3 could be encapsulated into a redox-sensitive nanogel using the inverse emulsion method and recovered under reductive conditions in the cytosol of the human cervical cancer (HeLa) cells, leading to the cancer cell apoptosis.

Traceless protein release is an emerging tendency in the design of protein-loaded nanogels because of the intact structure is important for the therapeutic efficacy of protein drugs. Free sulphydryl groups or primary amino groups of cysteine or lysine residues on proteins are commonly used for chemical modification, which could be linked to polymers via bioresponsive linkers to construct the stimulus-responsive prodrug polymers. The specific stimuli triggered the cleavage of linker and the release of protein drugs, while the released protein drugs may contain certain extra moieties from the broken linker, which may affect the bioactivities of the proteins. Traceless protein drug release strategy solved this problem, which was incorporated with a reversible linker that can undergo self-immolation. Inspired by the fact that free thiols are elevated in the activated T cells, Tang et al. developed a nanogel in response to T cell receptor (TCR) activation. First, they synthesized a bis-N-hydroxy succinimide (NHS) modified crosslinker (NHS-SS-NHS) containing a disulfide bond. The NHS on the linker further reacted with amino groups on IL-15 super-agonist complex (IL-15Sa), an adjuvant for increasing the response of adoptive cell transfer therapy. This reaction could be controlled to form nanogels. The GSH-triggered degradation of nanogels led to the release of intact IL-15Sa, which remained the protein activity. To the site-specific release of IL-15Sa in the solid tumor, the nanogel was anchored to the T cells as a backpack strategy. Using the receptor screening, only the CD45 receptor was found not to cause the internalization of the ligand-modified liposomes. The anti-CD45 antibody was incorporated to form the anti-CD45 functionalized nanogels, which could bind to the CD45 receptor on the T cells but did not affect the TCR signaling. Furthermore, the introduction of PEG-PLL maximized the efficiency of the nanogels because the positive charge of PLL promoted the electrostatic interaction with the T cells, which was beneficial for the ligand and receptor binding. This cytokine-loaded nanogel retained on the surface of inactivated T cells for more than 7 days, while IL-15Sa was rapidly released from the nanogels once the T cells were activated. Compared with free IL-15Sa plus exogenous pmel-1 T cells, the T cell backpack with IL-15Sa-containing nanogels did not activate the T cells in the circulation at equivalent dose, resulting in no systemic toxicity. Meanwhile, this backpack strategy increased the specific T cell expansion by 16 times than the free IL-15Sa in the solid tumor. The IL-15Sa nanogels were also applied to the human chimeric antigen receptor T cells, which eradicated tumors in mice at an efficiency of 80%.

In addition, this cell backpack strategy could also be used to suppress allogeneicity. Low dose of cytokine IL-2/Fc is essential for stimulating regulatory T cells (Tregs), which is critical in preventing unwanted immune response, such as acute or chronic graft rejection. Eskandari et al. prepared an IL-2/Fc-loaded nanogel with reduction sensitivity. The existence of reduction substance led to the traceless release of IL-2/Fc. This nanogel was then assembled to the surface of Tregs via the CD45 receptor-ligand interaction, which is same to T cells backpack with IL-15Sa above. The nanogel-engineered Tregs reduced the rejection in mice undergoing skin transplantation and prolonged their survival.

Chen et al. prepared a GSH-responsive nanogel loaded with a SARS-CoV-2 spike protein (S-RBD) by crosslinking S-RBD with NHS-SS-NHS. S-RBD is a subunit vaccine, which prevented the entry of the coronavirus into the cells with high expression of human angiotensin converting enzyme 2. However, S-RBD had low immunogenicity and poor pharmacokinetics, leading to inadequate uptake and processing by the dendritic cells or macrophages. The nanogel assisted S-RBD to accumulate the lymph nodes, where it could be efficiently taken up by the dendritic cells and macrophages. The high concentration of GSH in the dendritic cells and macrophages promoted the release of S-RBD, which activated the potent protective immune response to SARS-CoV-2. This nanogel-based subunit vaccine was genetic engineering-free, and provided the promise of a rapid, safe, effective, and economical vaccine preparation method.

5 | ENZYME-RESPONSIVE NANOGELS

Enzyme as a biological catalyst regulates the chemical reactions of metabolic processes in living organisms. The genesis and development of many diseases is related to the enzyme malfunction and expression dysregulation. The elevation of enzyme expression in the lesion could be
used as a pathological signal to design enzyme-responsive nanoparticles for drug delivery,\textsuperscript{25,43} such as matrix metalloproteinases (MMP) overexpressed in atherosclerosis\textsuperscript{63} and thrombin upregulated in thrombus.\textsuperscript{64}

Mesenchymal stem cells (MSCs) have the potential to differentiate into osteoblasts. Evidence showed that MSCs expressed fibrinolytic substances, such as plasmin and urokinase plasminogen activator.\textsuperscript{65} Kader \textit{et al.} reported a plasmin-responsive nanogel for enhanced differentiation from MSCs to osteoblasts.\textsuperscript{66} Plasmin-cleavable peptide (amino acid sequence: GGKFKTG) was incorporated as a linker into the amphiphilic polymer, which self-assembled into the nanogels in aqueous solutions. Bone morphogenetic protein-2 was conjugated to the nanogels. On-demand release of bone morphogenetic protein-2 from the nanogels enhanced the osteogenesis of MSCs.

Generation of oxidized low-density lipoproteins (oxLDL) within subendothelium leads to the accumulation of macrophages, which ingest the oxLDL and transform into foam cells.\textsuperscript{67} This process finally induced the deposition of atherosclerotic plaque that leads to many vascular diseases. MMP is upregulated in atherosclerosis. Based on these findings, Basak \textit{et al.} developed an MMP-2-responsive nanogel to deliver paraoxonase-1 (PON-1) for the treatment of atherosclerosis.\textsuperscript{68} PON-1 is an anti-inflammatory and antioxidizing enzyme, which prevents the accumulation of oxLDL and promotes the efflux of cholesterol from the macrophages. The nanogel was constructed by polymerization of acrylamide and PEGylated MMP-2-degradable peptide (GALGLP) crosslinker. The nanogel responded to the elevated MMP-2 and released PON-1 to reduce the level of ox-LDL, leading to the reduced transformation of macrophages to the foam cells.

Recently, our group proposed a close-looped hirudin delivery strategy using a self-regulated nanogel for the treatment of thrombosis.\textsuperscript{69} Thrombin is a critical protease in thrombosis, which transfers fibrinogen into fibrin to stabilize the blood clot. The nanogel was prepared using the inverse emulsion polymerization approach, which was composed of polyacrylamide network containing a thrombin-degradable peptide crosslinker (LVRPGS) and modified with a clot-targeted peptide ligand (CR(NMe)EKA). The recombinant hirudin was encapsulated in the obtained nanogel. The nanogel protected hirudin from proteolysis, and prolonged the circulation time of hirudin with increased bioavailability. After efficiently accumulating in the clot with the thrombin at a high concentration, the nanogel could responsively degrade and release the encapsulated hirudin. The released hirudin formed a stable complex by binding to the thrombin to produce anticoagulation activity for suppressing the clot formation. Meanwhile, the inactivation of thrombin inhibited its effect on degrading the nanogel, which rendered the recovery of the basal hirudin release and avoided the excess release leading to unexpected bleeding risk. Upon the thrombin elevated again under pathological stimuli, the above process could be repeated. We showed that this nanogel effectively prevented and inhibited the clot formation in the mouse pulmonary embolism and vascular thrombosis models.

6 SUMMARY AND OUTLOOK

With the development of genetic and protein engineering, more and more protein drugs have been applied for clinical treatment of a variety of diseases. The nanogel-based protein drug delivery systems prevent the unintended proteolysis of protein drugs, prolong their half-lives, and enhance their bioavailabilities. Site-specific protein drug release from the nanogels in an on-demand manner shows great potential on increasing the therapeutic efficacies and reducing the adverse effects. The difference in the biosignals between the lesion and normal tissues has been employed to design and formulate the bioresponsive nanogels, including temperature, acidity, redox potential, and enzyme expression.

Despite the considerable achievements in the bioresponsive nanogel-mediated protein delivery, there are still many challenges remaining to be addressed. The physical encapsulation and chemical coupling are two commonly used strategies for loading of the protein drugs. The physical encapsulation maintains the original structure of the proteins, but accidental protein leakage reduces the efficacies of the drugs.\textsuperscript{70} The chemical coupling reduces the protein leakage, and however, the structural modification may cause the impaired activity or inactivation of the protein drugs. The traceless protein release is a favorable strategy.\textsuperscript{71} The chemically coupled protein drugs can be released from the nanogels with their original structure under specific stimulation, which balances the dilemma of the physical encapsulation and chemical coupling strategy. Meanwhile, the multiresponsive nanogels have also been developed for more efficient protein release, while the complicated material/carrier synthesis for more functions limits their further generalization.\textsuperscript{72,73} Furthermore, due to the complexity of the body in vivo environment, it is difficult to control the endogenous biosignals, such as acidity or GSH, which are changing in different individuals. In addition, the easy-to-fabrication approach is required for the bioresponsive material/carrier development and further industrial manufacture, and the safety, metabolism, excretion, and efficacy should be comprehensively studied for clinical translation.\textsuperscript{74}
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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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