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Drug-Free Macromolecular Therapeutics – A New Paradigm in Polymeric Nanomedicines

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Abstract: This review highlights a unique research area in polymer-based nanomedicine designs. Drug-free macromolecular therapeutics induce apoptosis of malignant cells by the crosslinking of surface non-internalizing receptors. The receptor crosslinking is mediated by the biorecognition of high-fidelity natural binding motifs (such as antiparallel coiled-coil peptides or complementary oligonucleotides) that are grafted to the side chains of polymers or attached to targeting moieties against cell receptors. This approach features the absence of low-molecular-weight cytotoxic compounds. Here, we summarize the rationales, different designs, and advantages of drug-free macromolecular therapeutics. Recent developments of novel therapeutic systems for B-cell lymphomas are discussed, as well as relevant approaches for other diseases. We conclude by pointing out various potential future directions in this exciting new field.

1. Introduction

Macromolecular therapeutics, also referred to as polymeric nanomedicines, are a diverse group of drugs characterized by their large molecular weight (MW), including polymer-drug conjugates, polymeric micelles, polymer-modified liposomes, *etc*. The advantages of macromolecular therapeutics when compared to low-molecular-weight compounds are reviewed elsewhere.[1–3] In particular, water-soluble polymeric drugs (MW > 40 kDa) attain prolonged plasma half-lives and achieve tumoritropic accumulation due to the enhanced permeability and retention (EPR) effect.[4,5] Conventional polymeric nanomedicines utilize polymers as delivery vehicles to carry anticancer therapeutic agents. Many of these approaches are under clinical development.[6–12] Increasingly the role of nanomedicine is not only to deliver a given drug to diseased tissues efficiently but also to trigger or improve therapeutic effects through innate biological responses.[13,14] The design of macromolecular therapeutics has extended towards a unique paradigm where biomimetic strategies are employed to incite or control specific cellular activities.[15–17] For instance, receptor coupling (or clustering) can be used to sensitize diseased tissues to a therapeutic agent.[18–21] In this review, we highlight a novel paradigm in the nanomedicine research area – drug-free macromolecular therapeutics. This approach was firstly proposed by our laboratory in 2010.[21] The basic idea is to induce apoptosis by crosslinking of cell-surface non-internalizing receptors mediated by the biorecognition of high-fidelity natural binding motifs, such as antiparallel coiled-coil peptides or complementary oligonucleotides. The general design concept of drug-free macromolecular therapeutics is shown in Fig. 1. An important feature of these designs is the absence of low-molecular-weight cytotoxic compounds (thus named “drug-free”). This paper discusses recent developments in this exciting new area, which mainly includes research performed in our laboratory using B-cell malignancies as a

disease model and the CD20 receptor as a pharmacological target, as well as relevant approaches reported by other researchers.

1.1. B-cell lymphoma and CD20

Non-Hodgkin's lymphoma (NHL) is a prevalent cancer with over a half-million individuals having a history in the United States and an estimated 70,800 new cases diagnosed in 2014.[22] Over the past 3 decades, the incidence of NHL has continuously increased (doubled since 1980). NHL has a high mortality rate; from 2006 to 2010, there were 18,990 deaths for every 100,000 patients in the U.S.[22] The disease is comprised of a diverse and heterogeneous group of lymphatic malignancies, which makes the treatment challenging. About 85% of NHLs are cancers originating from B-cells; the remaining diseases are mostly of T-cell origin.[23] This review focuses mainly on designs and developments of novel therapeutics against B-cell lymphomas (or B-NHLs), including Burkitt's, diffuse large B-cell, follicular, immunoblastic large cell, precursor B-lymphoblastic, and mantle cell lymphomas. These malignancies are generally classified as either indolent or aggressive, which then dictates the type of therapy the patient may receive.[23,24] Besides conventional chemotherapy and radiotherapy, which are usually accompanied by severe adverse reactions, monoclonal antibodies (mAbs) targeted to the B-cell surface antigen CD20 have become common treatments.[25] Such "immunotherapies" have revolutionized the field. The current standard of B-NHL treatment is rituximab (the most commonly used anti-CD20 mAb) in combination with chemotherapy.[26,27] However, large populations of patients exist who do not respond or develop resistance to these therapies. For example, the overall response rates for the treatment of relapsed/refractory low-grade or follicular NHL typically ranged from 40 to 50% (complete response 6, 3, 17, 3, and 14%; overall

response 48, 46, 47, 39, and 43% in five different clinical trials).[28] The nonresponsiveness and/or resistance have been attributed to the inability of immune effector cells (*e.g.*, macrophages, natural killer cells) to hypercrosslink ligated mAbs,[29,30] and Fc receptor (FcR)-mediated endocytosis[31] or "trogocytosis"[32] of CD20 antigens. These clinical obstacles create the need for new, improved therapeutic strategies.

CD20 is a 35–37 kDa integral membrane protein highly expressed on more than 95% of B-cell lymphomas.[33,34] Free CD20 antigen is not present in serum, and there is no known natural ligand of CD20. When bound by antibodies, CD20 has a very low intracellular internalization rate;[35,36] it is often considered a non-internalizing receptor. Studies suggest that CD20 functions as a store-operated calcium channel and a cell cycle regulator.[37–39] It is one of the most reliable biomarkers of B-lymphocytes, thus providing an ideal target for treatment of B-NHL.[23,24] CD20 is also expressed on normal B-cells; however, it is not expressed on stem cells or progenitor cells and mature or activated plasma cells.[33] Therefore, the "B-cell depletion" therapeutic approach is considered safe; normal numbers of B-cells can be restored after treatment.[25–27] The therapeutic efficacy of anti-CD20 mAbs is ascribed to three cellular events: antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and CD20-mediated apoptosis.[40–42] All of these mechanisms require immune effector cells to function.[41] In contrast, drug-free macromolecular therapeutics trigger direct and specific apoptosis of B-cell lymphomas without the help of effector cells. This is achieved by the design of synthetic effectors that reproduce the function of immune effector cells. The advantages of such an approach will be further discussed in this review.

1.2. Receptor crosslinking and apoptosis

Cell receptor clustering (crosslinking) is a natural process and driving force for numerous biological responses. For instance, the following cellular events have been reported to result from receptor clustering: hormone uptake,[43] cell adhesion,[44] cell activation[45] and apoptosis.[42,46] In particular, crosslinking of the surface antigen CD20 induces apoptosis of B-cells. Research have shown that when CD20-bound antibodies are hypercrosslinked by FcR-expressing immune effector cells (or polyclonal secondary antibodies), CD20 receptors tend to cluster as dimers or tetramers, redistribute and become localized into lipid rafts.[47] Such events mediate the interaction between clustered CD20 and Src-family kinases (which are also located in lipid rafts), and trigger apoptotic signaling.[48,49] Without the hypercrosslinking, apoptosis initiated by ligated mAbs is limited.[50–52] These mechanistic studies warranted various earlier designs of multivalent mAb constructs. For example, Ghetie *et al.* synthesized a homodimer of rituximab by using a heterobifunctional crosslinker and showed that the mAb dimer potentiated apoptosis in human B-cell lymphomas, which synergized with a chemotherapeutic agent and an immunotoxin.[51] Rossi *et al.* produced a hexavalent anti-CD20 antibody by covalently assembling 6 Fab' to 1 Fc.[53] Anti-lymphoma efficacy of this hexavalent construct in mouse xenografts was comparable to that of the monovalent mAb, but it was independent of effector mechanisms such as CDC. Stein *et al.* used a monomeric Ab that lacks effector cell functions hypercrosslinked by a secondary Ab to specifically facilitate apoptosis.[54] These previous research showed that approaches aiming at direct apoptosis induction via cell surface receptor clustering are becoming attractive.

2. Origin of drug-free macromolecular therapeutics

The initial design of drug-free macromolecular therapeutics was inspired by our previous work on hybrid hydrogels self-assembled from synthetic polymers and coiled-coil protein domains.

We developed "smart" biomaterials composed of *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers grafted with biorecognition domains.[55–58] The biorecognition of complementary grafts resulted in physical crosslinking of polymer chains and formation of 3D networks (hydrogels). In particular, a pair of oppositely charged pentaheptad peptides (CCE and CCK) that form antiparallel coiled-coil heterodimers were designed (Fig. 2). Multiple copies of CCE or CCK were grafted to the HPMA polymer (P) backbones to produce P-$(CCE)_x$ and P-$(CCK)_y$, respectively. Equimolar mixtures of P-$(CCE)_x$ and P-$(CCK)_y$ solutions self-assembled into hydrogels where the coiled-coil peptides served as macromolecular physical crosslinkers.[58,59] The excellent CCE/CCK biorecognition was also employed by Lv *et al.* for the development of tandem modular protein-based hydrogels.[60] On the other hand, our laboratory pioneered the design of HPMA copolymers as anticancer drug carriers,[61,62] which led to the development of PK1 (HPMA copolymer–doxorubicin conjugate), the first polymeric drug that entered clinical trials.[63] HPMA copolymers are water-soluble, biocompatible, and long circulating in the bloodstream.[3,64] They have flexible (random-coil) conformation in aqueous solutions; thus, targeting moieties or biorecognition motifs that are grafted to the side chains can be effectively presented.[65] Based on these studies[58,59] and the above-mentioned mechanism of receptor clustering mediated apoptosis, we hypothesized that the unique biorecognition of the CCE/CCK peptide motifs could be used to crosslink not only polymer chains but also cell surface receptors (*e.g.*, CD20) to induce apoptosis of target cells (*e.g.*, B-cell). Such an application of hybrid materials to biological systems to mediate specific cellular events (*i.e.*, apoptosis) provides a bridge between the designs of biomaterials and novel nanomedicines.

3. Design of drug-free macromolecular therapeutics

3.1. Design based on formation of antiparallel coiled-coil peptides

Coiled-coils are common structural motifs in proteins where two or more right-handed α-helical peptides wind together to form a left-handed super-helix.[66] The primary structure of the coiled-coil motif is characterized by a sequence of repeating seven-amino-acid residues (heptad) designated as $[a, b, c, d, e, f, g]_x$; *a* and *d* are usually hydrophobic amino acids while the other residues are often polar.[67,68] Each peptide first folds into an α-helix, and the hydrophobic residues present as a "stripe" that coils around the helix to form an amphipathic structure. The hydrophobic interface then occurs between two helices, making *b*, *c*, and *f* face outward. Interhelical ionic interactions (between *e* and *g*) further stabilize (or destabilize) the coiled-coil conformation. These specific intermolecular interactions offer a high degree of structural control based on primary sequences. Consequently, coiled-coil peptides have become attractive as a building block for nanomedicine design.[58,69–71] We pioneered the development of drug-free macromolecular therapeutics, which employed a pair of 35-amino-acid coiled-coil forming peptides (CCE/CCK; see Fig. 2) as the biorecognition motif.[21] Two macromolecular conjugates were synthesized: (1) CCE attached to a Fab' fragment of anti-CD20 1F5 mAb (Fab'-CCE); (2) an HPMA copolymer grafted with multiple CCK peptides (P-$(CCK)_y$). Exposure of a $CD20^+$ human B-NHL cell line (Raji) to the Fab'-CCE conjugate first decorated the cell surfaces with CCE. Further treatment of the decorated cells with P-$(CCK)_y$ resulted in formation of antiparallel coiled-coils at cell surfaces, which crosslinked CD20 receptors and induced apoptosis.

3.1.1. *In vitro* and *in vivo* efficacies

The concept of drug-free macromolecular therapeutics was firstly proven by Wu *et al.* with the above-mentioned Fab'-CCE/P-$(CCK)_y$ CD20-crosslinking system *in vitro*[21] and *in vivo*.[72] The

coiled-coil formation was characterized by circular dichroism (CD) spectroscopy, and the biorecognition of the two conjugates occurred at the B-cell surface (Fig. 3A). Successful apoptosis induction of Raji cells was achieved after co-treatment with Fab'-CCE and P-$(CCK)_y$, either consecutively or as a premixture (Fig. 3B). The apoptosis-inducing activity, under different conditions, was comparable to or better than a mouse anti-CD20 mAb (1F5)[73] hypercrosslinked with a goat anti-mouse (GAM) secondary antibody.[21] *In vivo* anticancer efficacy of this novel system was further evaluated in mice bearing systemically disseminated B-NHL.[72] Both the consecutive (C) and the premixed (P) treatments were able to eradicate lymphoma cells in the blood and in the bone marrow, which produced long-term survivors (Fig. 3C).

3.1.2. Imaging studies

To study *in vivo* targeting of the Fab'-CCE/P-$(CCK)_y$ system, we recently performed multimodality imaging at the whole-body, tissue, and cellular levels.[74] Excellent cell surface biorecognition was observed in the spine, femur, tibia, liver, and spleen of mice, which are common "hot spots" of B-NHL dissemination.[75,76] After the first treatment with Fab'-CCE, high accumulation of P-$(CCK)_y$ was found within these lymphoma-enriched tissues (Fig. 4A). In contrast, mice injected with only P-$(CCK)_y$ (no Fab'-CCE) did not have such favorable tumor uptake. Whole body FMT (fluorescence molecular tomography) imaging confirmed the co-localization of signals indicating tumors, Fab'-CCE, and P-$(CCK)_y$, respectively. To elucidate the mechanism(s) involved in the apoptosis induction, plasma membrane lipid rafts of Raji cells were counterstained with a marker (AF555-CTB).[74] Under normal condition, lipid rafts spread throughout the plasma membrane, resulting in the AF555-CTB signal diffusion in a random punctate staining pattern on the cell surface (Fig. 4B, left panel). However, the consecutive

treatment with Fab'-CCE and P-$(CCK)_y$ disrupted normal lipid distribution on the cell surface and caused the formation of several intense fluorescent spots (indicating lipid raft clusters), which co-localized with patches of the two conjugates (Fig. 4B, right panel). These studies suggested that the apoptosis was indeed mediated by CD20 clustering (in lipid rafts) as a result of the Fab'-CCE/P-$(CCK)_y$ biorecognition.

3.1.3. Immunogenicity

Despite excellent efficacy of the Fab'-CCE/P-$(CCK)_y$ system, potential immunogenicity of the peptide conjugates is a concern before clinical applications. Short α-helical peptides are usually weak immunogens, unless administered with adjuvants.[77] Similarly, short peptides attached to HPMA copolymers have a minimal immunostimulatory response.[78] However, longer peptides (up to 40 residues)[79] can be immunogenic, especially when attached to macromolecular carriers to act as haptens.[80] Immunogenicity may change upon self-assembly,[81,82] which might result in the production of conformation-specific antibodies.[83,84] We evaluated the potential of individual peptides (L- and D- CCE, CCK), coiled-coils (CCE + CCK) and polymer conjugates (P-$(CCK)_y$) to activate RAW264.7 macrophages *in vitro*.[85] RAW264.7 cells were cultivated together with the tested compounds, and cytokine production was determined by ELISA (TNF-α, IL-1□, IL-6 and IL-10), and viability or changes in the surface markers (M1 vs. M2 polarization, activation markers) by flow cytometry. Lipopolysaccharide (LPS) served as the positive control of activation (and M1 shift). Neither HPMA copolymer nor any peptide, either L- or D-, induced any response in murine macrophages. The component responsible for macrophage activation was the 1F5 mAb or its Fab' fragment.

We further tested the *in vivo* immunogenicity of the conjugates in mice.[85] *In vivo* the therapeutics based on L-peptides (MIX L = Fab'-L-CCE + P-(L-$CCK)_y$) did not induce

substantially different Ab response than those based on D-peptides (MIX D = Fab'-D-CCE + P-(D-CCK)$_y$). The titer and avidity of Ab induced by i.v. treatment with MIX L or MIX D were generally low, slightly lower in the case of MIX D, except for anti-Fab'-CCE IgM Ab. In general, there were detectable Abs, but no cellular response to the therapeutics administered intravenously. Intravenous injection of Fab'-CCE, as well as the mixture of Fab'-CCE and P-(CCK)$_y$, triggered humoral immune responses, which led to the production of Abs directed against the Fab' part of the therapeutics. Nevertheless, P-(CCK)$_y$, when administered alone, was immunocompatible in mice. The major component responsible for the immunogenicity was again identified as the 1F5 mAb or its Fab' fragment.[85]

The biocompatibility and immunocompatibility of HPMA copolymers have been widely proven.[78,86–88] HPMA copolymers with oligopeptide side chains behaved as thymus-independent antigens with very limited immunogenicity and no mitogenic activity.[78] In clinical trials, patients were administered up to 30 g (in 6 infusions; 3 weeks apart) of HPMA copolymer-doxorubicin conjugates that contained GFLG tetrapeptide sequences, and no immunogenicity-associated side effects were observed.[63] In addition, it has been shown that conjugation of immunogens such as peptides or antibodies to HPMA copolymers could reduce the immunogenicity.[87,88] Therefore, we anticipate a favorable safety profile of the P-(CCK)$_y$ conjugate in the clinical setting. Since the immunological response was predominantly directed against Fab', which was from a mouse mAb (1F5), it is likely that Fab'-CCE will be immunostimulatory in humans. Interestingly, Press *et al.* treated 4 patients with the 1F5 mAb and observed minimal treatment toxicities.[73,89] Thus, immunogenicity may be acceptable for translation; however, to be on the safe side, humanization of the Fab' fragment is recommended before clinical applications. Alternatively, a system composed of human anti-CD20 mAbs (ofatumumab, veltuzumab, *etc.*) can be used.[90]

3.2. Design based on hybridization of morpholino oligonucleotides

The biorecognition of the coiled-coil forming oligopeptides, CCE and CCK, in the "drug-free" system worked well both *in vitro*[21,74] and *in vivo*.[72] However, to achieve a strong anticancer effect (produce tumor-free long-term survivors), we used a 1:25 molar ratio of CCE equivalent (in Fab'-CCE) to CCK equivalent (in P-$(CCK)_9$).[72] This is because the individual peptide sequences (CCE and CCK) do not have a pronounced secondary structure at pH 7 and are in a random coil conformation.[58] Binding of oligopeptides to macromolecules increases their secondary structure only slightly.[58,91] Consequently, Fab'-CCE and P-$(CCK)_y$ interact first via hydrophobic and electrostatic interactions, and then the oligopeptides fold into a strong antiparallel coiled-coil heterodimer. Such relatively complex binding pattern likely results in inadequate interaction of polymer conjugates with Fab' conjugates when administered at the 1:1 molar ratio condition. Therefore, we tried to identify a biorecognition pair that would bind efficiently at the 1:1 molar ratio. Morpholino oligonucleotides have been selected due to their fast hybridization, excellent binding affinity and stability in plasma as well as water-solubility.

Nucleic acid hybridization is a crucial biorecognition event in life. A DNA double helix is composed of Watson-Crick base paring, *i.e.*, hydrogen bonding of A/T and C/G, between two single-stranded polynucleotides with complementary sequences. The conformation is further stabilized by base stacking, *i.e.*, π-π interaction of neighboring bases on the same strand. Such a self-recognition property plays the central role for coding, storing and transferring of genetic information; it possesses high fidelity feature. Since early 1980s, DNA has been used as building blocks for biomaterials design,[92,93] and more recently, functional nanostructures for drug

delivery.[94,95] In particular, hybrid materials comprising oligonucleotides and synthetic polymers can be utilized to fabricate nanoconstructs with precise geometry and versatile functionality.[96–98]

Over the years, a variety of artificial oligonucleotides with chemically modified backbones have been synthesized.[99] These nonphosphodiester backbones are nuclease resistant and stable in the body; thus, they are suitable for biopharmaceutical applications. We designed a pair of phosphorodiamidate morpholino (MORF) oligomers, MORF1 and MORF2 (Fig. 5), as the biorecognition motifs for the second-generation "drug-free" therapeutic system.[100] The MORF oligos are charge neutral, resulting in significantly stronger binding than natural DNA and RNA.[101] Hybridization of the MORF pair has well-defined binding specificity, which prevents potential off-target effects.[102,103] In addition, MORF oligos have good aqueous solubility and favorable pharmacokinetics.[104,105] The sequences of MORF1 and MORF2 were designed to achieve optimal binding efficiency and minimal off-targets with human and murine mRNA, and to prevent self-complementarity.[100] This new therapeutic system was composed of two hybrid conjugates: (1) anti-CD20 Fab' linked to MORF1 (Fab'-MORF1), and (2) HPMA copolymers grafted with multiple MORF2 (P-$(MORF2)_x$). The two conjugates self-assembled via MORF1-MORF2 hybridization at the surface of $CD20^+$ B-cells, which crosslinked CD20 and initiated apoptosis.[100]

3.2.1. *In vitro* improvement

The efficacies of this new binary system (Fab'-MORF1/P-$(MORF2)_x$) was shown by Chu *et al*.[100] *In vitro* characterization by dynamic light scattering demonstrated that the two nanoconjugates, when mixed together at physiological conditions, rapidly reached a maximal binding within 10 min (Fig. 6A). In contrast, the CCE/CCK coiled-coil formation required a much longer time (~60 min).[21] Further analysis with UV-visible and CD spectroscopy indicated

that such binding was indeed mediated by MORF1/MORF2 hybridization and that the melting temperature was about 59 °C, well above body temperature. These results suggested a fast and stable self-assembly of the two conjugates, which are favorable for the design of drug-free macromolecular therapeutics. Importantly, when cell surface biorecognition and apoptosis were evaluated in Raji cells, we found that the treatment with **equimolar MORF1/MORF2** was sufficient to achieve substantial efficacies in this hybridization system (Fig. 6B).[100] However, for the coiled-coil design, a 25-time excess of the second peptide (CCE:CCK=1:25) was used.[21] Table 1 shows the side-by-side comparison of apoptosis induction between the two designs. Apoptotic index (%) of Raji cells was assessed under identical cell number and concentration; MW of the polymer backbones was both ~100 kDa. These data indicate that the oligonucleotide system induced higher levels of apoptosis when compared to the peptide system. Such phenomenon was observed in both the consecutive and the premixed treatment regimens. Comparison between the two systems in these designs suggests superior binding and accessibility of the MORF oligos on the HPMA polymer chains as compared to the coiled-coil forming peptides.

3.2.2. *In vivo* improvement

To evaluate *in vivo* anticancer efficacy of the hybridization system and to compare it with the previous design, we performed animal experiments using the same mouse model of systemic human B-NHL (Fig. 6C).[100] Mice were intravenously injected with Raji cells, followed by administration (i.v.) of the two conjugates. Results showed that, at equivalent doses, a single treatment of Fab'-MORF1 and P-$(MORF2)_x$ (MORF1:MORF2=1:1) was significantly more effective than a single treatment of Fab'-CCE and P-$(CCK)_y$ (CCE:CCK=1:25) on preventing lymphoma dissemination and on extending the animal survival (Table 1). The efficacy can be

further improved by using a 5-time excess of P-$(MORF2)_x$ (MORF1:MORF2=1:5).[100] Moreover, the time lag in the consecutive treatment can be optimized based on biodistribution and pharmacokinetics of the Fab'-MORF1 conjugate.[106] The comparison between the coiled-coil and the oligonucleotide designs clearly indicates that the hybridization system is advantageous for the drug-free approach. This is likely due to a more direct and specific binding pattern of the oligonucleotide base pairing at physiological conditions, when compared to the binding of the peptides, CCE and CCK. In addition, the charge neutral property of MORFs may help to prevent potential off-targets. These results indicate that, to improve therapeutic outcomes of drug-free macromolecular therapeutics, it is important to select a biorecognition pair with high binding efficiency.

3.2.3. Evaluation in patient samples

We evaluated the drug-free approach in chronic lymphocytic leukemia (CLL) cells isolated from 10 patients.[107] Primary cells were treated with Fab'-MORF1 and P-$(MORF2)_x$, and apoptosis and cytotoxicity were observed in 8 samples, including 2 samples with the 17p13 deletion. Chromosome 17p deletions are associated with the loss of one allele of the p53 gene, which portend an ultrahigh-risk prognostic factor.[108] The data suggest a p53-independent mechanism of apoptosis induction. This constitutes potential treatment for chemoresistant malignancies[109] and may synergize with other therapies.[110] Similarly, the approach also worked in cells from patients with mantle cell lymphoma, an aggressive subset of B-NHL that is particularly difficult to treat.[106] When compared to anti-CD20 mAbs 1F5 and rituximab, drug-free macromolecular therapeutics showed significantly more potent apoptosis-inducing activity and cytotoxicity. These results highlight the promising potential of the drug-free approach for clinical translation, as novel treatments against NHL, CLL, and other B-cell associated malignancies.

3.3. Other approaches

Another strategy is to use multivalent polymer-mAb or polymer-Fab' conjugates for direct CD20 crosslinking and apoptosis induction. For instance, multimeric rituximab bound to activated dextran[111] or lipid nanoparticles[112] have been produced. Our laboratory synthesized multivalent anti-CD20 Fab' attached to HPMA copolymer backbones, which successfully induced apoptosis of malignant B-cells.[113–115] The advantage of these approaches is the more straightforward one-step treatment, likely resulting to better patient compliance. However, due to the large size of the antibodies or their fragments, it is difficult to synthesize such constructs with high valency (due to steric hindrance). This undesirable feature may drastically limit the therapeutic efficiency. Previously we attempted to increase the valence by using branched polymer backbones[113,114] or linear, high-MW copolymers synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization.[115] Other researchers used biological polymers such as DNA or polypeptides as scaffolds to attach antibodies or the smaller size single-chain variable fragments (scFv).[17,116] These polyvalent constructs indeed achieved better efficacies than their monovalent counterparts. In addition, other targeting moieties such as aptamers have been employed for the crosslinking of different receptors, *e.g.*, CD30 (for Hodgkin's lymphoma)[117] and HER2 (for breast and gastric cancers).[118] Another approach is to use HPMA copolymers grafted with coiled-coil forming peptides and attach a complementary peptide terminated either in an anticancer drug or a single chain fragment as a targeting moiety.[70,71,119-121] Nevertheless, one fundamental difference between these single-treatment designs and the aforementioned binary systems is that the binary systems have the opportunity of performing pretargeting. This significant advantage will be further discussed in the next section.

4. Advantages of drug-free macromolecular therapeutics

The most important feature of drug-free macromolecular therapeutics is the lack of low-MW cytotoxic compounds and, thus, the absence of non-specific toxicities. The apoptosis induction is highly specific against the targeted cells, which will likely result in a better adverse effects profile when compared to conventional chemo- and radiotherapies. The mechanism of receptor crosslinking is unique. For instance, the CD20-clustering-mediated B-cell death has been identified as a distinct pathway that can bypass mitochondria and caspase activation, which offers the opportunity to treat chemoresistant malignancies.[109] Besides the drug-free feature, other favorable aspects are: (1) the proposed "two-step" treatment is suitable for pretargeting; (2) multivalency of the polymer conjugates has potential to improve therapeutic performance; (3) the immune-independent feature addresses the concern of mAbs nonresponiveness or resistance. The following subsections will discuss these advantages in details.

4.1. Pretargeting

The proposed two-step approach, *i.e.*, consecutive administration of Fab'-MORF1 (or Fab'-CCE) followed by P-$(MORF2)_x$ (or P-$(CCK)_y$), offers the opportunity of pretargeting. The pretargeting strategy is commonly used in cancer radioimmunotherapy.[122,123] The purpose is to achieve desirable pharmacokinetic goals by separating therapeutic modalities (*e.g.*, radionuclides) from targeting functionality (*e.g.*, antibodies). For instance, tumors have been pretargeted with an antibody conjugated to a MORF oligo; after a time lag to clear out **nonspecific binding**, radiolabeled complementary MORF oligos (therapeutic effectors) were administered for radiotherapy.[124,125] Over the years, the concept of pretargeting has been expanded and applied for

such strategies as amplified therapeutic delivery[126] and universal targeting of different tumor ligands.[127] These approaches aim to improve therapeutic efficacies and reduce adverse side reactions. Similarly, for drug-free macromolecular therapeutics, the Fab' conjugates can be used as a pretargeting agent, and then the multivalent polymer conjugates are delivered as the therapeutically active dose. The time lag between the two doses can be adjusted based on pharmacokinetics and biodistribution of the Fab' conjugates, in order to optimize pretargeting efficiency and achieve maximum tumor-to-tissue accumulation in individual patients. We have recently proven this concept in mice.[106] This was achieved by, first, finding a time lag when the pretargeting agent (Fab'-MORF1) was mostly cleared from the blood and reached a steady plasma concentration, and, second, by determining the tumor targeting efficiency when using this time interval. Results indicated a suitable timing for P-$(MORF2)_x$ administration at 5 h (in female SCID mice); at this time, Fab'-MORF1 was efficiently distributed to the tumors. Based on this result, we further performed therapy experiments in a disseminated B-NHL mouse model. When the optimized pretargeting time lag (5 h) was used, the therapeutic efficacy was significantly better than that of identical experimental conditions but with a 1 h interval. A low dose (58 μg × 3) of Fab'-MORF1 with a 5× excess P-$(MORF2)_x$ resulted in significantly delayed tumor growth and substantially improved animal survival.[106] The optimized therapeutic system surpassed rituximab in anticancer efficacy and completely eradicated lymphoma B-cells in 83% of the animals. This pretargeting approach may constitute a novel personalized nanotherapy to enable more efficient treatment and limit potential side effects associated with off-target binding.

4.2. Multivalency

A significant advantage of drug-free macromolecular therapeutics is the multivalency of the polymer conjugates, *i.e.*, multiple peptides or oligonucleotides per polymer chain. The multivalent effect describes the simultaneous interaction of repeated binding moieties in one molecular entity. Such interaction is superior to the monovalent binding kinetically and thermodynamically.[128,129] It has been reported that the multivalency of anti-CD20 constructs can magnify binding affinity and apoptosis induction by several folds, when compared to their monovalent or divalent counterparts.[17,113,114,116] In our drug-free approach, P-$(CCK)_y$ or P-$(MORF2)_x$ with valences up to 9 or 10 have been synthesized (using ~100 kDa polymer backbones). These conjugates would have multimeric interactions with targets, which possibly accounted for their significantly better therapeutic performance than the divalent mAbs as observed by Chu *et al.*[106,107]. Previously we have shown that, in addition to the valence, the MW of the polymer backbone also had a positive influence on the efficiency of CD20 crosslinking and apoptosis *in vitro*.[115] In the body, HPMA copolymers with larger MW tend to circulate longer in the blood.[64,65] This characteristic is favorable for targeting blood cancers such as lymphomas. Based on these promising aspects, the efficacy of the drug-free design may be further improved by using polymer conjugates with higher valences and/or larger polymer backbones. One method to approach this is to synthesize multiblock backbone-degradable HPMA copolymers by RAFT polymerization.[130-133] The MW (and valence) of the polymer conjugates can be substantially increased, while the biocompatibility is maintained.

4.3. Immune-independency

Distinct from mAb-based immunotherapy, drug-free macromolecular therapeutics directly induce apoptosis in diseased cells without the need for immune activation. Successful treatment

with mAbs requires FcR-expressing immune effector cells (macrophages, neutrophils, natural killer cells, *etc.*) to recognize the Fc region of ligated antibodies and trigger immune responses such as ADCC or CDC.[40,41] However, a common clinical failure of immunotherapy is the inactivation of these effector mechanisms.[29,30] For instance, many rituximab nonresponders harbor polymorphism in the IgG FcR gene, which leads to the inability of effector cells to hypercrosslink mAbs that are bound to the surfaces of B-cells.[29] In the drug-free design, we used synthetic effectors to reproduce and enhance the function of immune effector cells. High-fidelity biorecognition pairs are introduced externally to replace the Fc–FcR binding. This approach may benefit patients who do not respond to immunotherapies, which constitute about half of all B-NHLs.[28] In addition, the Fab' conjugates (without Fc) are used for pretargeting. This is advantageous because various reported mechanisms attributed to the mAb resistance are directly or partly mediated by the Fc–FcR recognition, for example, CD20 downregulation,[134,135] internalization,[31,36] and "trogocytosis" (shaving of receptors from cell surfaces by macrophages).[32] The designed Fc-independent apoptosis induction may circumvent these mechanisms, resulting in a potential to target mAb-resistant diseases. Michel and Mattes have shown that the 1F5 mAb/CD20 complex becomes non-internalizing when the Fc region of the mAb is removed.[36] This observation further strengthens our point of view. Moreover, mAb therapies may "over-activate" the immune responses, which results in adverse side reactions, *e.g.*, hypersensitivity due to complement activation that requires discontinuation of treatment and administration of corticosteroids.[136] These side effects are sometimes fatal (*e.g.*, cytokine storm[137] and rituximab-associated lung injury[138,139]). In contrast, our direct apoptosis induction strategy does not rely on immune functions; this may ease such concerns. We have demonstrated that, at equivalent doses, the 2nd-generation (hybridization-mediated) drug-free design possesses

superior or comparable anti-lymphoma efficacies to type I anti-CD20 mAbs.[106] These data suggest significant advantages of the drug-free therapeutics paradigm over conventional immunotherapies. Mechanistic studies to compare drug-free macromolecular therapeutics with Type II mAbs (*e.g.*, obinutuzumab) which may also induce direct apoptosis[140,141] will provide further evaluation of the clinical potential.

5. Conclusions and beyond

In summary, drug-free macromolecular therapeutics constitute a new paradigm of polymer-based nanomedicines that are free of toxins and immune activation. Cell surface biorecognition of hybrid nanomaterials translates into innate biological responses, *i.e.*, apoptosis. The apoptosis induction is direct (without the help of effector cells) and specific (targeted to certain receptors) and suitable for the design of precisely pretargeted nanotherapies. This novel approach has significant advantages over conventional chemo-, radio-, and immunotherapies. These promising perspectives warrant further developments within the same pipeline and may stimulate other designs. Here, we suggest potential future directions and provide supporting literature for each direction:

1) *Targeting moieties*: peptide ligands identified by combinatorial methods,[142,143] oligosaccharides,[144,145] and oligonucleotide aptamers.[146,147] An aptamer for the B-cell receptor has been identified.[146] Bifunctional nucleic acids can be produced that contain aptamers (targeting moieties) and crosslinkers (binding motifs) on each end of one molecule.[147]

2) *Binding motifs*: different sequences and lengths (*e.g.*, longer motifs with spacers may result in less steric hindrance of binding[148]), other types of binders such as peptide nucleic acids

(PNA),[149,150] locked nucleic acids (LNA),[146,151] and 2'-*O*-methyloligoribonucleotides (2'-OMe-RNA).[151]

3) *Polymer backbones (or other carriers)*: liposomes,[152] carbon nanotubes,[126] or genetically engineered biopolymers (*e.g.*, polypeptides,[116] poly-DNA[17]). Mobility and biodistribution of carriers should be characterized. Flexible backbones are generally preferred for receptor crosslinking.

4) *Different diseases*: CD20 crosslinking and B-cell depletion can be used for autoimmune disorders such as rheumatoid arthritis,[153] multiple sclerosis,[154] and systemic lupus erythematosus.[155] The same approach can potentially be used for anti-rejection treatment of organ transplants, *e.g.*, rituximab is used off-label for kidney transplant recipients.[156]

5) *Cell receptors*: potentially any non- or slowly internalizing cell surface antigen can be a target, such as CD45 (T-cell, B-cell, macrophage),[157] death receptor 4 (breast and colon cancers, *etc.*),[158] prostate stem cell antigen (prostate cancer),[159] and carcinoembryonic antigen (many tumor types, but not on normal cells).[160,161] The crosslinking of these antigens can induce cell apoptosis.

6) *Other directions*: crosslinking two different receptors simultaneously to achieve synergistic effects (*e.g.*, CD20/CD40,[162] CD20/FGFR3,[163] CD37/CD20 or CD37/CD19[152]), and designed as a switch for ON-OFF regulation of cellular events.[158,164]

For further translation into the clinic, the drug-free therapeutic approach will ultimately require validation and confirmation in properly conducted clinical trials, as well as carefully designed *in vivo* biocompatibility/toxicity studies. For applications in cancer, the tumor penetration capability of each of the therapy components shall be evaluated. It will be interesting to compare the mobility of the nano-sized therapeutic conjugates with that of the immune

effector cells, which have limited penetration into solid tumors. In conclusion, we anticipate more designs and research in this exciting new field of polymeric nanomedicines.

Figure Legends:

Fig. 1 Drug-free macromolecular therapeutics for apoptosis induction. Crosslinking of cell surface non-internalizing receptors is mediated by the biorecognition of natural binding motifs. Two hybrid conjugates can be administered consecutively as pretargeting and crosslinking doses, or premixed to form a multivalent construct and used as a single dose.

Fig. 2 Helical wheel diagram of the CCE/CCK coiled-coil antiparallel heterodimer.[58] The heptad repeat of each peptide is labeled *a–f*. Both CCE and CCK were modified with a YGG peptide spacer (to prevent steric hindrance of binding after grafted to polymer chains) and functionalized with a cysteine (for conjugation).

Fig. 3 Coiled-coil based drug-free macromolecular therapeutics. (A) Cell surface biorecognition of Fab'-CCE (labeled with rhodamine; red) and P-$(CCK)_9$ (labeled with FITC; green). Raji B-cells were exposed to the mixture of the two conjugates and imaged by confocal fluorescence microscopy within 4 h. (B) *In vitro* apoptosis induction of Raji B-cells as analyzed by annexin V/propidium iodide assay. Cells were treated with Fab'-CCE and P-$(CCK)_9$, or 1F5 mAb hypercrosslinked by goat anti-mouse (GAM) 2° Ab. Control (ctrl) groups are as indicated. P-NH_2: polymer precursor of P-$(CCK)_9$. Data are presented as mean ± SD (n = 3). (C) *In vivo* therapeutic efficacy against systemic B-NHL. Four million Raji cells were injected via the tail vein of SCID mice (n = 7 per group), which induced hind-limb paralysis. *Control*: untreated. *CS*: consecutive, single-dose. *PS*: premixed, single-dose. *CM*: consecutive, multiple-doses. *PM*: premixed, multiple-doses. Paralysis-free animal survival is presented in a Kaplan-Meier plot. Numbers of long-term survivors are indicated. Figures are adapted from Wu *et al.*, 2010[21] and Wu *et al.*, 2012.[72]

Fig. 4 Multimodality imaging of coiled-coil based drug-free macromolecular therapeutics. The Fab'-CCE conjugate was labeled with FITC, and P-$(CCK)_9$ with Cy5. (A) *In vivo* biorecognition as characterized by fluorescence molecular tomography (FMT) imaging. Raji B-cells were stained with the DiR dye and intravenously injected to mice. One day later, mice were administered (i.v.) Fab'-CCE and P-$(CCK)_9$, as a premixture (Premix) or consecutively (Cons) using different time lags (1 h or 4 h). Tumor-inoculated mice injected with P-$(CCK)_9$ only served as controls. *L*: liver. *K*: kidney. (B) Three-dimensional z-stack confocal images showing distribution patterns of lipid rafts on the surfaces of Raji cells. Cells were treated consecutively with the two conjugates. Non-treated cells served as controls. Circular regions indicate lipid rafts clusters. Graticule size: 10 μm. Figures are adapted from Zhang *et al.*, 2014.[74]

Fig. 5 Structure and base sequences of the morpholino oligonucleotide pair, MORF1 (8630.5 Da) and MORF2 (8438.5 Da).[100]

Fig. 6 Oligonucleotide hybridization mediated drug-free macromolecular therapeutics. (A) Effective hydrodynamic diameters of the two conjugates and their mixture (equimolar MORF1/MORF2; different times after mixing) as characterized by dynamic light scattering. Statistics, unless otherwise indicated, was performed by comparing the mixture with P-$(MORF2)_3$. (B) Apoptosis of Raji B-cells as analyzed by annexin V assay. *Consecutive*, Fab'-MORF1 followed by equimolar P-$(MORF2)_3$; *Premixed*, equimolar mixture of Fab'-MORF1 and P-$(MORF2)_3$; *mAb + 2° Ab*, 1F5 mAb followed by goat anti-mouse secondary Ab. Statistics, unless otherwise indicated, was performed by comparing each group with the untreated cells. (C) Therapeutic efficacy against systemic B-lymphoma in SCID mice (n = 6–8 per group). Four million Raji cells were injected via tail vein on day 0. *Cons ×1*, consecutive treatment of equimolar Fab'-MORF1 and P-$(MORF2)_{10}$, 1-dose; *Prem ×1*, equimolar mixture of Fab'-MORF1 and P-$(MORF2)_{10}$, 1-dose; *Cons (1:5) ×1*, consecutive treatment, MORF1:MORF2 = 1:5, 1-dose; *Cons ×3*, 3 doses of consecutive treatment; *Prem ×3*, 3 doses of premixture; *1F5 mAb ×3*, 3 doses of 1F5 mAb. One-dose treatment on day 1; three doses on days 1, 3 and 5. Statistics was performed with the log-rank test. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0001$, *n.s.*: no significant difference. Figures are adapted from Chu *et al.*, 2014.[100]

Tables:

Table 1 Comparison of *in vitro* and *in vivo* anti-B-NHL efficacies between the coiled-coil based and the oligonucleotide hybridization mediated drug-free macromolecular therapeutics.

In vitro* – Apoptotic Index				
	CCE/CCK	MORF1/MORF2		
Consecutive	(1 μM, valence=9)	(0.5 μM, valence=3)	(1 μM, valence=3)	(0.5 μM, valence=9)
	12%	23%	37%	50%
Premixed	(1 μM, valence=9)	(0.5 μM, valence=3)	(1 μM, valence=3)	(0.5 μM, valence=9)
	16%	17%	39%	43%
***In vivo* – Median Survival Time**[†]				
	CCE/CCK	MORF1/MORF2		
Consecutive	(1 nmol, 1:25)	(1 nmol, 1:1)		
	50 days	81 days		
Premixed	(1 nmol, 1:25)	(1 nmol, 1:1)		
	55 days	78 days		

*Apoptotic index (%) of Raji cells assessed by annexin V assay. Concentrations of Fab' and valences of polymer conjugates are listed; comparison at time intervals corresponding to maximum apoptosis. Data are from Wu *et al.*, 2010[21] and Chu *et al.*, 2014.[100]

[†] Median survival (day) of mice bearing systemic B-cell lymphoma and exposed to different treatments. Data are from Wu *et al.*, 2012[72] and Chu *et al.*, 2014.[100]

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Conflict of interest

J.K. and T.-W.C. are inventors on a pending US patent application (PCT/US2014/023784; assigned to the University of Utah) related to drug-free macromolecular therapeutics. J.K. is Chief Scientific Advisor for Bastion Biologics. Otherwise, the authors declare no competing financial interests.

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Figure 1

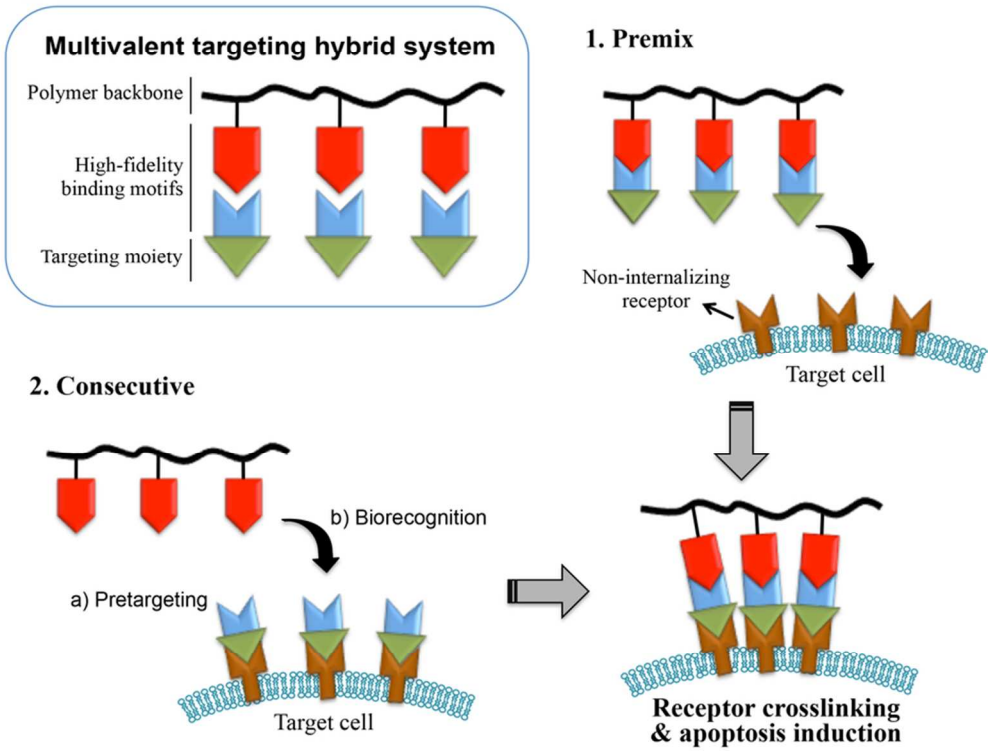

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Figure 2

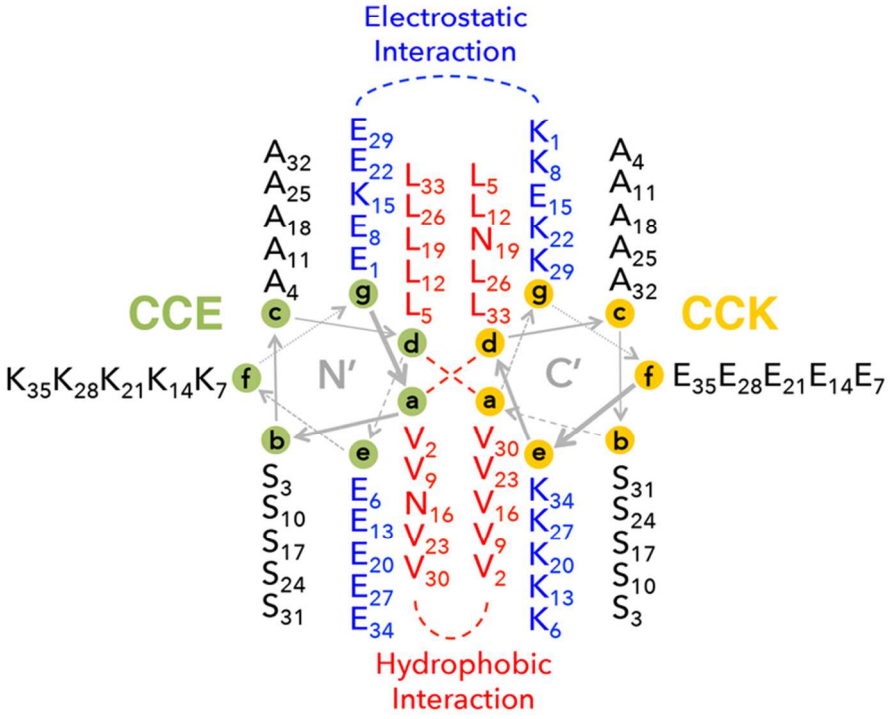

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Figure 3

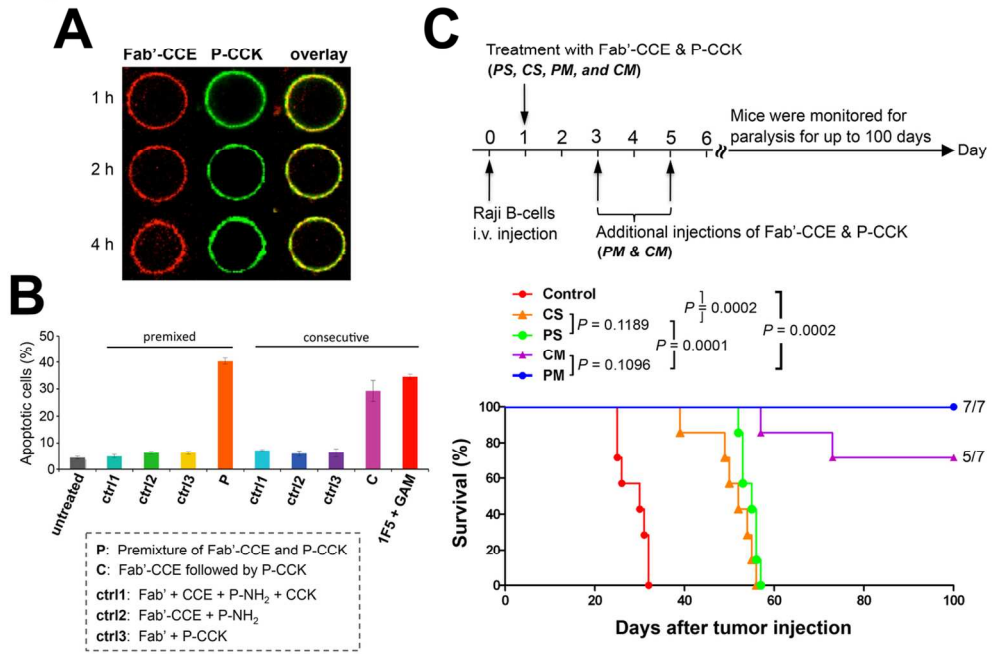

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Figure 4

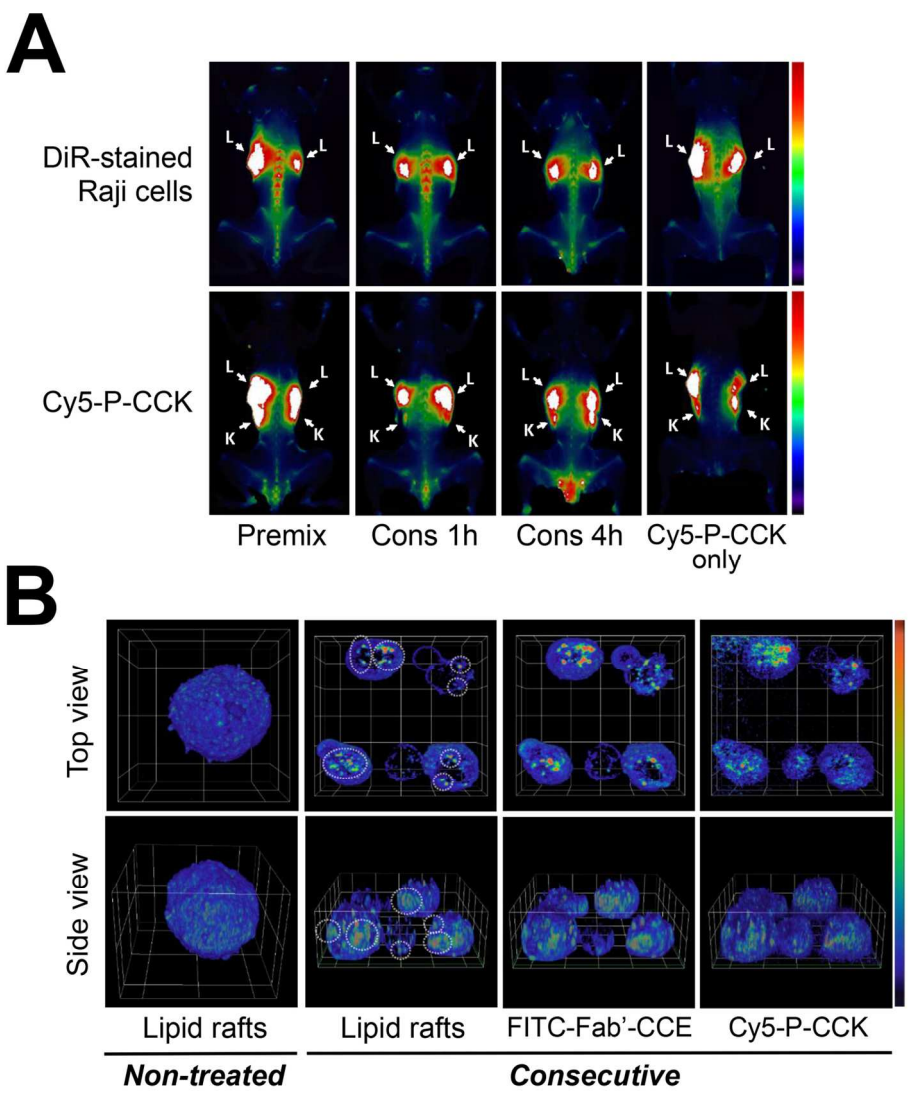

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Figure 5

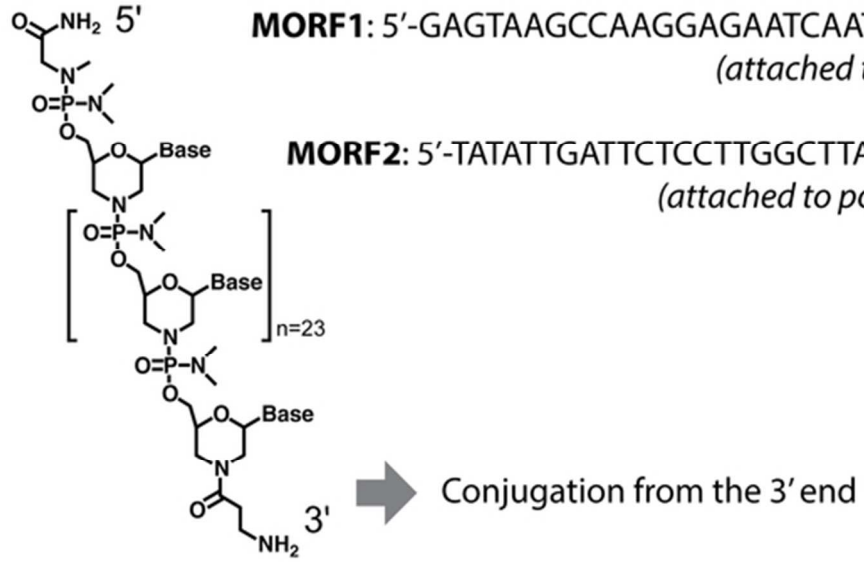

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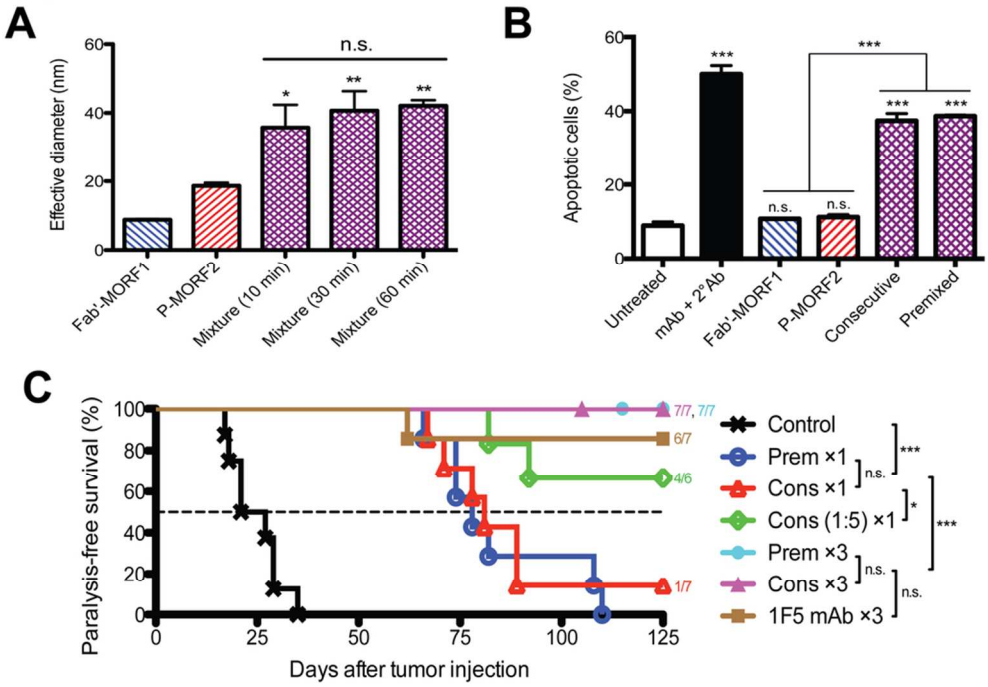
Figure 6



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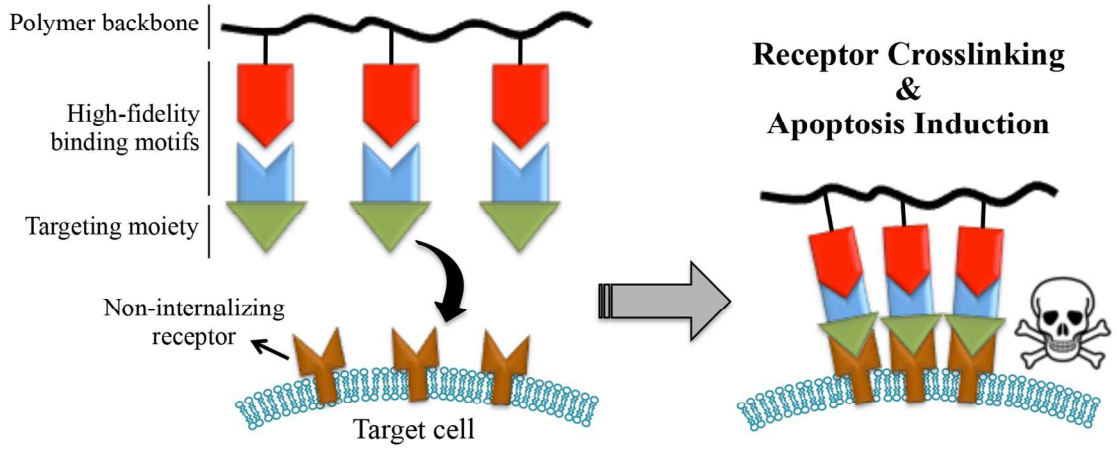

This review highlights an exciting new field of polymeric nanomedicine research – drug-free macromolecular therapeutics for cell apoptosis induction.