Published in final edited form as:

Adv Healthc Mater. 2015 May ; 4(7): 1054–1065. doi:10.1002/adhm.201400679.

Multimodality Imaging of Coiled-Coil Mediated Self-Assembly in a “Drug Free” Therapeutic System

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Abstract

We used two complementary coiled-coil peptides CCE/CCK to develop a “drug free” therapeutic system, which can specifically kill cancer cells without a drug. CCE was attached to the Fab’ fragment of anti-CD20 1F5 antibody (Fab’-CCE), and CCK was conjugated in multiple grafts to poly[N-(2-hydroxypropyl)methacrylamide] (P-(CCK)_x). Two conjugates are consecutively administered: First, Fab’-CCE coats peptide CCE at CD20 antigen of lymphoma cell surface; second, CCE/CCK biorecognition between Fab’-CCE and P-(CCK)_x leads to coiled-coil formation, CD20 crosslinking, membrane reorganization, and ultimately cell apoptosis. To prove that two conjugates can assemble at cell surface, multiple fluorescence imaging studies were performed, including 2-channel FMT, 3D confocal microscopy, and 4-color FACS. Confocal microscopy showed co-localization of two fluorescently labeled conjugates on non-Hodgkin’s lymphoma (NHL) Raji cell surface, indicating “two-step” targeting specificity. The fluorescent images also revealed that these two conjugates could disrupt normal membrane lipid distribution and form lipid raft clusters, leading to cancer cell apoptosis. This “two-step” biorecognition capacity was further demonstrated in a NHL xenograft model, using fluorescent images at whole-body, tissue and cell levels. We also found that delaying injection of P-(CCK)_x could significantly enhance targeting efficacy. This high-specificity therapeutics provide a safe option to treat NHL and other B cell malignancies.

Keywords

coiled-coils; CD20 crosslinking; lipid raft clusters; non-Hodgkin's lymphoma; N-(2-hydroxypropyl)methacrylamide (HPMA)

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Author contributions: R.Z., J.Y., and J.K. designed research; R.Z., J.Y., T.W.C., and J.H., performed research; R.Z. and J.K. analyzed data; and R.Z. and J.K. wrote the paper.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

1. Introduction

In nature, protein heterodimerization mediated by coiled-coils can lead to the formation of either an “active” or an “inactive” multiple-protein complex, thereby switching molecular pathways “on” or “off” in many cellular processes.^[1] Inspired by this principle, we developed a coiled-coil-mediated switch which can turn on apoptosis pathways in cancer cells with a high degree of specificity. As one of the most frequently encountered biorecognition motifs in nature, coiled-coils are present in ~4% of all amino acids of proteins and peptides,^[2] and in up to 10% of eukaryotic proteome.^[3] Since nature has provided us with such perfect motif for molecular recognition and assembly, much attention has been attracted to the applications of self-assembling coiled-coil peptides for engineering functional materials, such as multimeric antibodies, epitope display, affinity purification, tissue engineering, regenerative medicine, biosensor, nanomaterials assembly, and drug delivery.^[4-18] In our group, two complementary coiled-coil peptide sequences (CCE and CCK) were previously designed and successfully assembled hybrid graft copolymers into hydrogels.^[19,20] Recently, this coiled-coil pair was employed in a therapeutic system to specifically trigger apoptosis in cancer cells.^[21,22] In this new system (**Figure 1**), one peptide (CCE) was attached to the Fab’ fragment of anti-CD20 1F5 antibody (Fab’-CCE), and the complementary peptide (CCK) was conjugated in multiple grafts to poly[N-(2-hydroxypropyl)methacrylamide] (HPMA) (P-(CCK)_x; P is the HPMA copolymer backbone). As designed, the treatment is given in two steps: In the 1st step, the Fab’-CCE conjugate is administered to label CD20 antigens on cancer cell surface; Then, the P-(CCK)_x conjugate is applied in the 2nd step to crosslink those Fab’-CCE-prelabeled antigens. Our “two-step” approach differs from “two-step” pretargeting in nuclear medicine,^[23,24] though both employ antibody-related conjugates to pre-label tumor cells in the 1st step. Instead of small-molecule radionuclide or drug, we employ a hybrid graft copolymer in the 2nd step to crosslink specific antigens on cancer cell surface, re-organize plasma membrane domains, and turn on apoptosis signaling pathways. Previously, our group tested this new therapeutic for the treatment of Non-Hodgkin lymphoma (NHL) and it showed a great anti-tumor potential *in vitro*^[21] and *in vivo*^[22]. If either conjugate was absent, no cytotoxicity was observed.

In our system, CD20 antigen (Cluster of Differentiation Antigen 20) was selected for specific targeting in the 1st step. CD20 is a hydrophobic transmembrane protein expressed by mature B cells and most malignant B cells, but not by stem cells, pre-B cells, normal plasma cells or other normal tissue cells.^[25] It is not shed from the cell surface,^[26] not internalized upon antibody binding,^[27] and not found in the circulation.^[28] Thus, CD20 is considered an ideal target for antibody-mediated therapy and has been successfully used in clinics for the treatment of B-cell NHL, chronic lymphocytic leukemia (CLL), and autoimmune diseases.^[29-32] Although the endogenous role of CD20 is still not fully defined, it has been found to function through binding to Src family tyrosine kinases (i.e., Lyn, Fyn, and Lck) and involve in phosphorylation cascade of intracellular proteins.^[33-35] Ligation of CD20 with antibodies can result in the formation of signaling microdomains (lipid rafts), and eventually cause calcium flux and activation of caspases.^[36] To date, CD20-targeted therapeutics have made an improvement in the treatment of immune-related diseases.

However, there are still some major drawbacks, such as lack of specificity (up to 50% non-responders), high toxicity, and serious adverse effects. Therefore, our system was designed to significantly increase therapeutic specificity *via* a chain of actions (Figure 1). - a) pre-labeling of CD20 antigens with anti-CD20 Fab' fragment coupled to an affinity system; b) biorecognition of complementary sequences and formation of coiled-coil heterodimers; c) crosslinking of CD20 antigens by polymeric chain and induction of apoptosis. Ultimately, only antibody fragment binding in concert with crosslinking of CD20 antigens can trigger cell death.

Obviously, our system kills cancer cells on a distinctive principle as compared to currently used therapeutics. This new treatment does not involve any small-molecule drug or toxin, and also individual components do not possess cytotoxicity.^[21] Thus, we named it “drug-free macromolecular therapeutics”. However, our previous understanding of its crosslinking and *in vivo* “two-step” assembly was all derived from indirect evidence, such as apoptosis onset and tumor inhibition.^[21,22] In this work, we employed multiple imaging techniques to obtain a deep insight into how this new system performs its function, particularly in an animal mode (Figure 1). The images exhibited “two-step” assembly of our macromolecular system on the targeted cancer cells at whole-body, tissue and cell levels.

2. Results

2.1. Preparation of biorecognizable conjugates

Figure 1 shows the sequences of the coiled-coil forming peptides, CCE and CCK. Their N-termini were modified with functional groups (maleimido for CCE and thiol for CCK, respectively) for conjugation. The formation of coiled-coils by CCE/CCK peptides was previously determined using circular dichroism spectroscopy.^[19,20] The Fab' fragment from a mouse anti-human CD20 IgG2a antibody (1F5) was tethered to CCE-mal *via* a thioether bond to produce a Fab'-CCE conjugate with molecular weight of ~55 kDa (**Figure 2**). To prepare HPMA copolymer-CCK conjugate (Figure 2), we first synthesized HPMA copolymer containing side chains terminated in amino groups by reversible addition-fragmentation chain transfer (RAFT) polymerization, followed by reaction with succinimidyl-4-(*N*-maleimido-methyl)cyclohexane-1-carboxylate (SMCC) to produce side chains terminated in maleimide groups.^[37] Then, CCK-sh peptide was grafted *via* a stable thioether linkage to the side chains of the HPMA copolymers. The final product P-(CCK)₉ had 9 CCK grafts per macromolecule, and its molecular weight was about 160 kDa. To follow *in vivo* behavior of conjugates with optical imaging, we fluorescently labeled both conjugates: FITC-labeled Fab'-CCE (FITC-Fab'-CCE) and Cy5-labeled P-(CCK)₉ (Cy5-P-(CCK)₉), as described in Figure 2.

2.2. Assembly of two conjugates induces lipid raft clusters at cancer cell surface

Although previous circular dichroism and dynamic light scattering results showed that Fab'-CCE and P-(CCK)₉ conjugates, when mixed in solution, self-assembled and formed coiled-coil heterodimers,^[21] it is necessary to demonstrate that FITC-Fab'-CCE and Cy5-P-(CCK)₉ can specifically assemble at CD20 antigens on the cell surface. Thus, we conducted *in vitro* confocal microscopy study, using CD20-expressing human NHL Raji B cell line (**Figure**

3a). First, exposure of Raji B cells to FITC-Fab'-CCE resulted in decoration of cell surface with FITC signal (green), because of CD20 antigen-Fab' biorecognition. Further incubation of FITC decorated cells with Cy5-P-(CCK)₉ resulted in co-localization of Cy5 signal (red) with FITC on the cell surface (Figure 3a). If an excess amount of 1F5 antibody or CCK peptide was also added in the consecutive treatment, there was less Cy5 signal, due to a blocking effect (Figure 3a). If the cells were treated with Cy5-P-(CCK)₉ only (without any FITC-Fab'-CCE), Cy5 signal was also absent (Figure 3a). When two conjugates (FITC-Fab'-CCE and Cy5-P-(CCK)₉) were premixed for 1 h and then added to Raji B cells as a mixture, the fluorescence images showed co-localization of two signals. In summary, these microscopic results revealed that two conjugates could specifically assemble at CD20 antigens in two steps and Fab'-CCE was required for 2nd step binding.

The next question was whether the system was able to crosslink the CD20 antigens and reorganize plasma membrane as we designed. Thus, the Raji cells treated with two fluorescently labeled conjugates, were counterstained with a lipid raft marker Alexa Fluor 555 cholera toxin subunit B (AF555-CTB). Three-dimensional confocal microscopy was performed to observe the distribution patterns of three fluorochromes on entire plasma membranes. In general, lipid rafts spread throughout the cell membrane under normal condition.^[38-40] As shown in Figure 3b, weak AF555-CTB signal diffused in a random punctate staining pattern on the cell surface. After consecutive treatment, the conjugates disrupted homogenous distribution of fluorescence and caused several intense fluorescence spots on the cell surface, indicating formation of lipid raft clusters (Figure 3b). It has been reported that clustering lipid rafts can form membrane platforms, which can recruit or aggregate various receptors (i.e., tumor necrosis factor (TNF) α receptors, insulin receptors, and Fas) and also gather various signaling molecules (i.e., G-proteins, tyrosine kinases, phosphatases, and sphingomyelin), resulting in activation of cell signaling pathways.^[41-45] We also found that lipid raft clusters co-localized with patches of FITCFab'-CCE and Cy5-P-(CCK)₉ as well (Figure 3b), which might explain apoptosis induction by those two conjugates.^[21]

2.3. "Two-step" biorecognition and assembly *in vivo*

In our previous reports,^[21,22] this system not only killed cultured lymphoma B cells, but also inhibited tumor growth in SCID mice bearing B-cell NHL. Particularly, we found that two conjugates could cure the mice in a consecutive "two-step" way.^[22] To further confirm this finding, we conducted a series of *in vivo* and *ex vivo* imaging experiments to simultaneously track lymphoma cells and two conjugates after intravenous injection. This investigation took advantage of multiple imaging technologies, including fluorescence molecular tomography (FMT), *in vivo* imaging system (IVIS), confocal microscopy, and flow cytometry, in order to provide a clear picture of coiled-coil mediated self-assembly at whole-body, tissue and cell levels. Prior to *in vivo* evaluation, we had to define the time interval between two consecutive injections, because the efficacy of a "two-step" targeting approach depends on the injection timing. According to previous pretargeting reports in radiology,^[23,24] the 2nd-step conjugate should be administered at the time when the 1st-step antibody fragment conjugate reaches highest tumor-to-blood ratio. Therefore, we first analyzed the pharmacokinetic profile of the Fab'-CCE conjugate. In Figure S1 (Supporting

Information), blood radioactivity-time profile of ^{125}I -labeled Fab'-CCE in mice is illustrated and the pharmacokinetic parameters are summarized. Its initial half-life ($t_{1/2, \alpha}$) was 19.8 min, while terminal half-life ($t_{1/2, \beta}$) was 7.99 h. Because 1st-step Fab' conjugate was not internalized, the timing of 2nd-step administration could be delayed until unbound Fab'-CCE concentration in blood decreases to a low level. In this case, we found that the concentration of Fab'-CCE in the plasma reached a steady state or plateau at 4 h after intravenous injection, and then remained nearly constant over time. Therefore, the optimal time interval between 1st step and 2nd step was determined to be 4 h. In the following animal studies, a 1 h interval was used as a control.

At whole-body level—Firstly, we needed to locate the tissues where inoculated lymphoma cells are enriched. To this end, luciferase-expressing Raji cells were intravenously injected into SCID mice *via* tail vein. Three weeks after orthotopic inoculation, bioluminescence imaging showed that the Raji cells mainly lodged in several tissues including the femur, tibia, spine, liver, spleen, and lymph node (Figure S2, Supporting Information). After defining the location of inoculated lymphoma cells, we performed high-resolution FMT imaging to get an overview about the distribution of 2nd conjugate P-(CCK)₉ and lymphoma cells. The Raji cells labeled with XenoLight DiR fluorescence dye were intravenously inoculated into SCID mice *via* tail vein. One day later, the mice bearing systemically disseminated Raji cells were injected with FITC-Fab'-CCE and Cy5-P-(CCK)₉ either concurrently (pre-mixture) or consecutively. The mice treated with Cy5-P-(CCK)₉ only (without FITC-Fab'-CCE) served as a control. **Figure 4a** shows the whole-body fluorescence images of the mice 48 h after administration of Cy5-P-(CCK)₉. Similarly as found in aforementioned bioluminescence imaging (Figure S2, Supporting Information), DiR-labeled Raji cells accumulated in the spine, femur, tibia, liver, and spleen (Figure 4a and Figure S3, Supporting Information). After injection of both conjugates, no matter which strategy was used, there was a relatively high Cy5-P-(CCK)₉ uptake within those Raji-enriched tissues. In contrast, the mice only injected with Cy5-P-(CCK)₉ had less Cy5 signal in their skeleton (i.e., spine, femur, and tibia). In addition, we also found that Cy5-P-(CCK)₉ accumulated in the kidney (Figure 4a and Figure S3, Supporting Information).

At tissue level—After whole-body FMT imaging, major tissues were harvested immediately and their fluorescence signal intensities were measured using *in vivo* imaging system (IVIS) equipped with DiR and Cy5 filters. As shown in Figure 4b, the biodistribution data confirmed previous findings from FMT images. The majority of Raji cells were found in the liver and spleen. Additionally, there were a smaller number of Raji cells residing in the lung, femur, tibia, and lymph node. In those tissues, the mice treated with both conjugates had significantly higher Cy5-P-(CCK)₉ uptake than the mice injected with Cy5-P-(CCK)₉ only ($p < 0.05$) (Figure 4b). This remarkable difference further confirmed the indispensability of FITC-Fab'-CCE. To better compare targeting efficacy, we analyzed the fluorescence intensity ratio of Cy5-P-(CCK)₉ to DiR-labeled Raji cells (Figure 4c). In femur, tibia and lymph node, 4-h consecutive approach caused significantly higher delivery efficacy of Cy5-P-(CCK)₉ than the other three strategies ($p < 0.05$) (Figure 4c). This finding proved our previous hypothesis - delaying Cy5-P-(CCK)₉ administration to a time when

tumor-to-blood ratio of FITC-Fab'-CCE is most favorable, could lead to enhanced targeting efficacy in the 2nd step. In Figure 4c, we also found that there was no significant difference between 1-h consecutive and pre-mixture injection. It might explain why those two treatments showed a similar therapeutic outcome in a previous animal study.^[22]

At cellular level—To confirm conjugate assembly on lymphoma cells, we performed an additional experiment to investigate the cells inside the bone marrow. After biodistribution measurement, bone marrow cells were harvested from the hind limb (femur and tibia), and stained with Raji B-lymphoma marker, phycoerythrin (PE) anti-human CD10 antibody. A 4-color flow cytometry assay was performed with PE anti-CD10 antibody, DiR, FITC-Fab'-CCE, and Cy5-P-(CCK)₉ (Figure 4d). First, the majority of harvested cells showed PE-negative, while less than 1% cells were PE-positive (PE+). Not surprisingly, the small number of PE+ cells were also detected as DiR positive (DiR+), thereby confirming the presence of inoculated Raji B-lymphoma cells inside bone marrow of recipient mice. Next, we further analyzed the double-positive (PE+DiR+) cells. In this Raji cell fraction, 83.2% ± 6.5% of cells exhibited double positive staining at FITC-Fab'-CCE and Cy5-P-(CCK)₉ (n = 3) (Figure 4d). It indicated that most of targeted Raji cells were dual-labeled with two conjugates. For comparison, we also evaluated off-target delivery in normal bone marrow cells. In those double-negative (PE-DiR-) normal cells, there were only 0.002% ± 0.002% cells attached with both conjugates (FITC+Cy5+), while a few cells had single color (FITC+Cy5-: ~0.1%; Cy5+FITC-: ~1%) (Figure 4d). Those numbers revealed that consecutive treatment with the two conjugates had a high-degree targeting specificity in bone marrow.

To further confirm the specificity, we also used multi-channel confocal microscopy to separately track lymphoma cells and the conjugates at other organs. Since 4-h consecutive treatment already showed a superior targeting effect over the other two approaches, confocal microscopy will focus on Raji-enriched tissues under 4-h consecutive treatment, including lung, spleen, liver, and bone. Here, PE anti-CD10 antibody was used to pre-label inoculated Raji cells instead of DiR. At 2 d after conjugate administration, the tissues were harvested and observed under high-resolution confocal microscope. **Figure 5** shows representative confocal 3D z-stack images. A number of PE-labeled Raji cells were found in those excised Raji-enriched organs after intravenous inoculation. Most of these Raji cells (blue) displayed both FITC (green) and Cy5 (red) signal as well. The merged images showed a prominent co-localization of three signals (PE, FITC, and Cy5), which displayed as a white color (Figure 5 and Figure S4, Supporting Information). Due to limited penetration of FITC signal, the green intensity in deep tissues could be reduced so that some of cells exhibited purple color. In the FITC channel, there was some tissue autofluorescence. We also found Cy5 signal in normal liver and spleen cells. However, those non-specific uptake signals were much lower as compared to specific ones on Raji cells. In addition, we also took an image of calvarium (frontoparietal bone of the skull) where the thin bone is compatible with direct observation without damaging the tissues. We also used ultraviolet (UV) signal to provide outline of bone structure. As showed in Figure 5 and Figure S4 (Supporting Information), 3-color co-localization also occurred in calvarium, especially inside the hollow regions.

Similarly, *ex vivo* confocal images of the mice co-injected with a pre-mixture showed that Cy5-P-(CCK)₉ co-localized with FITC-Fab'-CCE at Raji cells in those tissues (**Figure 6**).

Taken together, those images exhibited a good correlation between Raji cells and two conjugates at the cellular level, which highlighted the targeting specificity of our therapeutics.

3. Discussion

To date, a major concern of all therapeutics in cancer and immune diseases is that they act indiscriminately, killing cancer or disease-associated cells, disease-irrelevant cells and various normal tissue cells simultaneously. Thus, we developed a new therapeutic system which is able to specifically re-organize cancer cell membrane microdomains and turn on apoptosis signaling pathways in cancer cells (Figure 1). Our previous data already showed that “drug free” macromolecular therapeutics could kill lymphoma cells without aid of drug or toxin *in vitro* and *in vivo*.^[21,22] However, the previous understanding of its performance was all derived from therapeutic effect. In this study, we used multiple imaging techniques to gain a deep insight into the mechanism of action. To some degree, our findings also highlight two major advantages of this unique system - high specificity and great flexibility.

Cells communicate with the outside environment through their membrane that interacts with extracellular stimuli (i.e., antibodies, hormones, other cells) and translates this information to intracellular responses. Changes in the organization of the membrane are critical to transmembrane signal transduction.^[44] It is known that the plasma membrane is not homogeneous but instead contains various microdomains, such as lipid rafts and protein clusters.^[41-45] It has been found that there is a 10-fold enrichment of signaling proteins in lipid raft microdomains versus the whole membrane.^[46] The lipid rafts are associated with some signaling molecules, such as Lck, Ras, Fyn, Grb-2, and phosphatidylinositol-3 kinase,^[47-51] suggesting their role as a signaling platform where close intermolecular proximity increases specificity and efficiency in the regulation of signaling pathways. Moreover, it is speculated that incidental combinations of different signaling molecules by lipid rafts might induce novel signaling and influence regular intracellular processes.^[52-54] Therefore, lipid rafts recently turned into a hot target for therapeutic intervention and we took advantage of this significance to develop a therapeutic system.^[21,22] As we expected, the confocal microscopy showed that our system could disrupt the normal distribution of lipid rafts and form lipid raft clusters *via* crosslinking of CD20 antigens (Figure 3b). It explains why we previously saw apoptosis onset in lymphoma cells treated with two conjugates.^[21] It also reveals that this therapeutic system performs its cytotoxic function only when crosslinking of Fab' fragments on CD20 antigen occurs at the cell surface. Based on this principle, this “two-step” system is able to kill cancer cells with a high degree of specificity, while sparing the normal cells. We were aware that the specificity to some extent depends on administration timing of 2nd-step graft copolymer conjugate. We tested three different administration strategies, including co-injection of pre-mixture, 1-h consecutive and 4-h consecutive injection. From the biodistribution results (Figure 4b and 4c), we found that 4-h consecutive treatment caused significantly higher Cy5-P-(CCK)₉ uptake at tumor sites than the other two. It was mainly attributed to the relatively high tumor-to-blood ratio of FITC-Fab'-CCE at 4 h post-injection. This finding suggested that delaying the administration of 2nd-step conjugate could increase therapeutic efficacy. In addition, a prolonged administration interval also could reduce risk of non-specific toxicity. Because

CD20 is not internalized after antibody binding,^[27] 1st-step Fab'-CCE conjugate which specifically binds to CD20 antigen will remain on cell surface, while Fab' conjugates which non-specifically attached on normal cell surface will enter the cells over the time. After a certain time period, P-(CCK)₉ will rarely have the possibility to perform crosslinking on normal cell surface, thereby significantly reducing non-specific toxicity. Even if there were a small number of Fab'-CCE remaining on normal cell surface, their crosslinking might not cause lipid raft clusters, due to the lack of lipid-raft relative binding sites. Thus, the consecutive approach has a great potential of improving therapeutic effect and safety profile. In contrast, injection of pre-mixture of both conjugates has no such advantages, because in this case a multivalent assembly containing many Fab' fragments will form prior to administration. Their large size would limit their penetration at tumor resident site (i.e., bone marrow). Therefore, as compared to the "two-step" pretargeting, "one-step" approach generally has lower treatment specificity and meanwhile higher non-specific toxicity risk.^[23,24] Results shown on Figure 4c confirm that premixed treatment had significantly lower targeting efficacy than the 4-h consecutive one. Additionally, those large-size macromolecules might cause some side effects and immune response.^[37] Previously, the polymer conjugates with covalent attachment of Fab' fragment were also developed by our group.^[55-57] Because of steric hindrance, It was difficult to conjugate multiple Fab' on one polymer chain. The limited number of Fab' grafts might make the conjugate not have sufficient crosslinking capability and also the large size had aforementioned safety concerns. Accordingly, we prefer to treat mice bearing systemically disseminated NHL B-lymphoma cells with Fab'-CCE and P-(CCK)₉ in a consecutive way.^[22] Recently, Pechar *et al.* developed a new strategy of using recombinant DNA technology to incorporate a coiled-coil peptide into a single chain (scFv) fragment.^[17,18] This fusion protein approach could produce a high amount of well-defined scFv-peptide conjugates directly. More importantly, the coiled-coil peptide will have a minimal effect on the structure and biological activity of the scFv fragment. However, the fusion protein might possibly have different conformations, resulting in altered function, lower activity or even inaccessibility of binding sites.

The key factor for this "two-step" treatment is to incorporate an affinity system, which makes assembly of two conjugates operative *in vivo*. In the past decades, a variety of affinity systems have been developed, such as biotin/streptavidin, bispecific-antibody/hapten, and complementary sequence pairs (peptide, DNA, RNA). However, only a few products were able to progress beyond an *in vitro* experimental stage and most of them were nonfunctional in further animal evaluation. Fortunately, our affinity pair CCE/CCK showed excellent biorecognition *in vitro* as well as *in vivo*, as demonstrated in our imaging studies. Moreover, the coiled-coil design allows this system to be one-size-fits-all models, which can be adopted for the treatment of other diseases. The antitumor (targeting) component of the 1st-step conjugate may be replaced with different types of antibody fragment or targeting ligands, thus aiming various diseases. For example, some receptors or other CD antigens, like immunoglobulin E (IgE) receptor,^[58] and T cell antigen receptor (TCR),^[59] etc, are also reported to be associated with similar membrane microdomains. Incorporation of such antibody fragment into our conjugate promotes the artificial formation of microdomain clusters into an organization where novel associations are created and signaling pathways

may be initiated. In this work, we exhibited a CD20-targeted NHL B-lymphoma therapy. NHL is the seventh most common cancer and seventh most common cause of cancer-related deaths in the United States. It is known that more than 90% of B-cell NHLs express CD20 antigen.^[28,29] Although Rituximab and other newer antibodies against CD20 have made significant improvements to NHL therapy, there are still more than 50% of patients with aggressive B-cell NHL incurable.^[60] We anticipate that this new class of therapeutics could transform the current situation. In addition, this new B-cell depletion therapeutic can also be used for the treatment of chronic lymphocytic leukemia (CLL), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), and other diseases as well.

4. Conclusions

In summary, our “drug free” macromolecular therapeutic performs an anti-cancer function on a distinctive principle. Only antibody fragment binding in concert with crosslinking of the expressed target antigens triggers cell death, a specific process with minimal toxicity to normal cells. Moreover, the consecutive treatment strategy enables preparation of conjugates “on request” for personalized therapy. Therefore, we believe that this new generation of therapeutics is going to become one of preferred concepts to treat cancer in the future.

Experimental Section

Reagents—Common reagents were purchased from Sigma-Aldrich and used as received unless otherwise specified. *N*- α -Fmoc protected amino acids were purchased from P3 Biosystems and AAPTEC. V501 (4,4'-azobis(4-cyanopentanoic acid)) was purchased from Sigma-Aldrich and V65 (2,2'-azobis(2,4-dimethyl valeronitrile)) from Wako Chemicals. Succinimidyl-4-(*N*-maleimido methyl)cyclohexane-1-carboxylate (SMCC) was purchased from Soltec Ventures. Chain transfer agent 4-cyanopentanoic acid dithiobenzoate (CPDB)^[61] and *N*-(2-hydroxypropyl)methacrylamide (HPMA)^[62] were synthesized as previously described. *N*-(3-Aminopropyl)methacrylamide (APMA) was purchased from Polysciences. All solvents were purchased from Sigma-Aldrich. Cyanine5 NHS ester was purchased from Lumiprobe and fluorescein-5-isothiocyanate (FITC) was from Life Technologies. Iodine-125 [¹²⁵I] was obtained from Perkin Elmer. XenoLight DiR fluorescent dye (DiIC18(7); 1,1'-dioctadecyltetramethyl indotricarbocyanine iodide) was purchased from Perkin Elmer. CD10 Mouse Anti-Human mAb (clone MEM-78) PE conjugate was purchased from Life Technologies.

Peptide synthesis—: As described in our previous reports,^[19-21,37] the peptides CCE (E VSALEKE VSALEKK NSALEKE VSALEKE VSALEK) and CCK (K VSALKEK VSALKEE VSANKEK VSALKEK VSALKE) were synthesized using Fmoc/tBu strategy and solid phase synthesis methodology on a PS3TM peptide synthesizer (Protein Technologies). Tripeptide YGG was added to each N terminus as spacer. To introduce the maleimide group to the N terminus of CCE (denoted as ‘CCE-mal’), the peptide was capped with heterocrosslinker SMCC at the last step. Similarly, CCK peptide with N terminus thiol modification (denoted as “CCK-sh”) was obtained by addition of a cysteine residue.^[19,20] The peptides were purified using RP-HPLC on a semipreparative column (Zorbax 300SB-C18, 250 × 9.4 mm, Agilent Technologies 1100 series), and were verified using MALDI-

TOF mass spectrometry (UltrafleXtreme, Bruker Daltonics) (Figure S5, Supporting Information).

Preparation of FITC-labeled Fab' conjugate—Fab' fragment was prepared as described in Figure 2a. The murine 1F5 anti-CD20 IgG2a antibody (Ab) was prepared by culturing a hybridoma cell line in a CellMax bioreactor (Spectrum Laboratories). Ab was harvested from the reactor and purified on a protein G Sepharose 4 Fast Flow column (GE Healthcare). Purified 1F5 Ab was then digested into F(ab')₂ using pepsin (10 wt %) in citric buffer (pH 4) for 2 h at 37°C. To introduce a fluorescent probe, F(ab')₂ was labeled with fluorescein isothiocyanate (FITC) in PBS (pH 7.4) at 4°C overnight. FITCF(ab')₂ was then purified using ultrafiltration to remove byproducts and unreacted dyes. To estimate the degree of labeling, the FITC-F(ab')₂ sample was analyzed by UV-Vis spectrophotometry (Varian). The amount of FITC per F(ab')₂ was calculated using the absorbance at 495 nm with an extinction coefficient of 82,000 cm⁻¹M⁻¹ in PBS. Purified labeled FITC-F(ab')₂ was reduced using 10 mM tris(2-carboxyethyl)phosphine (TCEP) in PBS for 1 h at 37°C. TCEP was removed using ultrafiltration. FITCFab'-CCE was prepared by thiol-ene reaction by adding CCE-mal to the FITC-Fab' solution in 10 × molar excess. Unconjugated peptides were removed using ultrafiltration. The conjugate FITC-Fab'-CCE, antibody 1F5, and antibody fragments (FITC-F(ab')₂ and FITC-Fab') were analyzed using fast protein liquid chromatography (FPLC), respectively (Figure S6a, Supporting Information).

Preparation of Cy5-labeled polymer conjugate—To prepare HPMA copolymer grafted with several copies of CCK peptide, a copolymer (P-NH₂) of HPMA and *N*-(3-aminopropyl)methacrylamide (MA-NH₂) was first synthesized by RAFT copolymerization using 4,4'-azobiscyanovaleric acid (V501) as the initiator, and 4-cyanopentanoic acid dithiobenzoate as the chain transfer agent (CTA) (Figure 2b).^[37] After polymerization, the copolymer (100 mg) was further reacted with 2,2'-azobis(2,4-dimethyl valeronitrile) (V65; 10 mg, over 40 times excess with respect to the polymer end groups) in MeOH (1 mL) at 50°C for 2.5 h. Following chain end modification, a white powder polymer was obtained by precipitation into acetone twice. The aqueous solution was scanned by UV-visible spectrophotometry and there was no detectable signal at ~300 nm wavelength, indicating the removal of dithiobenzoate end groups. The average molecular weight and the polydispersity of the conjugates were determined by size exclusion chromatography (SEC) on an AKTA FPLC system equipped with a UV detector (GE Healthcare), miniDAWN TREOS and OptilabREX (refractive index, RI) detector (Wyatt Technology) using a Superose 6 HR10/30 column with sodium acetate buffer containing 30% acetonitrile (pH 6.5) as mobile phase. The number average molecular weight was 121 kDa (Mw/Mn = 1.19). The amine content in the copolymer P-NH₂ was 307 nmol/mg (37 NH₂ groups per chain) as determined by ninhydrin test. To fluorescently label the copolymer conjugate, Cy5-NHS ester and copolymer P-NH₂ were dissolved in DMF (molar ratio of [Cy5]: [NH₂] = 1:2) and stirred at room temperature for 12 h. After coupling, the sample was purified using PD-10 Sephadex G25 column (GE Healthcare) to remove free dye. The amount of Cy5 per chain in the conjugate Cy5-P-NH₂ was determined by UV-visible spectrophotometry using the extinction coefficient of 250,000 cm⁻¹M⁻¹ at 646 nm (6 Cy5 molecules per chain). Then the remaining amino groups at side chain termini were converted to maleimide groups by

reaction with SMCC at room temperature for 5 h (molar ratio of [SMCC]: [NH₂] = 5:4). The maleimide content of the precursor was 187 nmol/mg (23 molecules of maleimide groups per chain) as measured by modified Ellman's assay. Finally, the CCK peptide was covalently attached to the copolymer by reaction of the thiol group on cysteine with the maleimide groups on the polymer backbone forming a stable thioether bond. The polymer and peptide were dissolved in PBS with 10 mM TCEP (molar ratio of [CCK-sh]: [maleimide] = 1:2) and stirred at room temperature for 16 h. After the coupling reaction, an excess amount (10×) of free cysteine was used to quench the unreacted maleimide groups on HPMa polymeric backbone following conjugation with CCK-sh. Six hours later the conjugate was dialyzed against DI water using ultrafiltration with molecular weight cutoff 30 kDa (to remove unreacted CCK-sh and cysteine) and then lyophilized. The average number of CCK grafts per chain was determined as 9 using amino acid analysis. A representative size exclusion chromatogram is on Figure S6b (Supporting Information).

Cell culture—Human Burkitt's B-cell NHL Raji cells (ATCC) were maintained at 37°C in a humidified atmosphere containing 5% CO₂ in RPMI-1640 medium (Gibco) supplemented with 10% FBS and a mixture of antibiotics (100 units/mL penicillin, 0.1 mg/mL streptomycin).

Confocal microscopy of cultured cells in vitro—Raji cells were treated with two fluorescently labeled conjugates (FITC-Fab'-CCE and Cy5-P-(CCK)₉) using "Premixed" and "Consecutive" strategies, respectively. For the "Premixed" treatment, FITC-Fab'-CCE and Cy5-P-(CCK)₉ were initially mixed in culture medium at the concentration of 0.4 μM at 37°C for 1 h and then 1×10⁵ Raji cells were incubated in 0.5 mL of medium containing the mixture for 1 h. For the "Consecutive" treatment, 1×10⁵ Raji cells were first exposed to 0.4 μM FITC-Fab'-CCE in medium (0.5 mL) at 37°C for 1 h; then, the cells were washed twice with PBS to remove unbound FITC-Fab'-CCE and treated with Cy5-P-(CCK)₉ in medium (0.5 mL) for another 1 h. In addition, an excess amount of 1F5 antibody (prior to FITC-Fab'-CCE) or CCK (prior to Cy5-P-(CCK)₉) was added into "Consecutive" treatment for blocking. The cells treated with Cy5-P-(CCK)₉ only (without pre-exposure of FITC-Fab'-CCE) served as a control. In parallel, a portion of cells following "Consecutive" treatment were additionally stained with Alexa Fluor-555 conjugated Cholera toxin B subunit (CTB) at 4°C for 30 min to investigate the influence on lipid-raft distribution pattern by treatment. After incubation, cells were washed 2 times with PBS and fixed with 4% paraformaldehyde. The cell samples were transferred onto glass bottom microwell dishes (MatTek) and visualized under Nikon A1R confocal fluorescence microscope equipped with a FITC filter (excitation/emission=488/520 nm), a Cy5 filter (excitation/emission=646/665 nm) and an Alexa Fluor-555 filter (excitation/emission=555/565 nm).

Cell labeling—To track Raji cells *in vivo* and *ex vivo*, labeling cells with fluorescent probes was conducted immediately before inoculation. For FMT and IVIS imaging, the Raji cells were stained with 10 μM DiR (1,1'-dioctadecyltetramethyl indotricarbocyanine iodide) at 37°C for 20 min, while R-phycoerythrin (PE)-labeled mouse anti-human CD10 was used to label cells according to the manufacturer's protocol for *ex vivo* confocal microscopy of tissues. Following staining, the cells were washed twice with cold PBS and prepared for

intravenous inoculation. Flow cytometry revealed an average labeling efficiency of ~95 % and no cytotoxicity at the dose of labeling (Figure S7, Supporting Information).

Tumor model—All animal studies were carried out in accordance with the University of Utah IACUC guidelines under approved protocols. Following the animal models described previously^[22], fluorescently labeled human Burkitt's B-cell NHL Raji cells (4×10^6) in 200 μ L of phosphate buffered saline were intravenously injected into female nude SCID mice (6- to 8- weeks old, 20–25 g, Charles River Laboratories) *via* tail vein.^[22]

Radiolabeling and pharmacokinetics study— Na^{125}I (Perkin Elmer) was added into Fab'-CCE conjugate PBS solution in pre-coated iodination tube, and gently stirred at room temperature for 10 min. After labeling, the sample was then purified with Sephadex PD-10 columns. The specific activity of the hot samples was in the range 1.5-2.0 mCi/mg. For the pharmacokinetic study, 6- to 8-week-old healthy female nude SCID mice (20-25 g; Charles River Laboratories) (n=5) were intravenously injected with ^{125}I -labeled Fab'-CCE (50 μ g, 20 μ Ci/mouse). At predetermined intervals, blood samples (10 μ L) were taken from the tail vein, and the radioactivity of each sample was measured with Gamma Counter (Packard). The blood pharmacokinetic parameters were analyzed using a two-compartment model with WinNonlin 5.0.1 software (Pharsight).

FMT imaging—At day 1, female nude SCID mice were intravenously injected with 4×10^6 DiR-labeled Raji cells in 200 μ L PBS *via* the tail vein. At day 2, inoculated mice were divided into 4 groups to receive different treatments (FITC-Fab'-CCE: 50 μ g/mouse; Cy5-P-(CCK)₉: 325 μ g/mouse) (n=3): a) Premix, co-injection of premixed two conjugates; b) Con-1h, consecutive administration of two conjugates at 1-h interval; c) Con-4h, consecutive administration of two conjugates at 4-h interval; d) Control, administration of Cy5-P-(CCK)₉ alone (without FITC-Fab'-CCE). Here, in the “Premix” group, the two conjugates (FITC-Fab'-CCE and Cy5-P-(CCK)₉) were first mixed at 37°C for 4 h, and then the mixture was intravenously injected *via* tail vein. Consecutive treatments involved the intravenous injection of FITC-Fab'-CCE first and 1 h (or 4 h) later Cy5-P-(CCK)₉ conjugate was given *via* the same route. At day 4, after the mice skin was removed to allow imaging of deep tissues, whole-body fluorescence imaging was performed using an FMT camera (Visen Medical) equipped with lasers (wavelength: 635 nm and 745 nm).

Biodistribution study—At the end of FMT imaging session, various tissues (i.e., blood, heart, liver, spleen, kidney, lung, muscle, femur, tibia, and lymph nodes) were harvested. The fluorescence signal of those tissues were measured using an IVIS 200 imaging system (Caliper Life Sciences) with the filters of Cy5 (excitation/emission: 605nm/666nm) and DiR (excitation/emission: 710nm/780nm). Identical illumination settings, including exposure time (5s), binning factor (small), f-stop (2), and fields of view (13×13 cm), were applied for all image acquisitions. Total emission from inflicted areas (Region of Interest, ROI) of each tissue was quantified with Living Image 4.0 software, and the average radiant efficiency [(photons/sec/cm²/sr)/(μ W/cm²)] was measured (n=3). Ratio of Cy5-P-(CCK)₉ conjugate to DiR-labeled Raji cells was calculated on the basis of fluorescent intensity values of the corresponding tissues (n=3).

Flow cytometry of residual Raji cells—After *ex vivo* measurement of tissues by IVIS, the bone marrow cells were collected by purging femurs with cold PBS. The harvested cells were incubated with red blood lysis buffer for 2 min and washed twice with PBS to remove cell debris. Then, the cells were stained with PE-labeled mouse anti-human CD10 at 4°C for 30 min in the dark and washed twice with cold PBS. Four-color flow cytometry analysis was performed using BD LSRFortessa machine (BD Biosciences). Cell percentages were analyzed using FlowJo software (Tree Star). Fluorescent composition of cells was performed using single staining with each fluorochrome separately. Compensation values were set to eliminate spectral overlap.

Confocal microscopy of tissues *ex vivo*— 4×10^6 PE-labeled Raji cells were intravenously injected into female nude SCID mice *via* the tail vein. One day after inoculation of cells, the mice received aforementioned “Premix” or “Con-4h” treatment. The mice were sacrificed 48 h after administration of conjugate Cy5-P-(CCK)₉. Immediately after harvest, the tissues, including lung, liver, spleen, and calvarium, were observed under Nikon A1R confocal microscopy equipped with a FITC filter (excitation/emission=488/520 nm), a Cy5 filter (excitation/emission=646/665 nm) and a PE filter (excitation/emission=488/575 nm).

Statistical analysis—Data were presented as mean $\pm$ standard deviation. Statistical analyses was done using a two-tailed unpaired Student's t-test, with p values of <0.05 indicating statistically significant differences.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Dr. Ruozhen Hu and Professor Ryan O'Connell for assisting with flow cytometry analysis, Brian Watson and Professor Edward W. Hsu for help with FMT imaging study, as well as Dr. Christopher Rodesch and Cell Imaging Core facility for confocal microscopy. We also thank Tian Yu for the assistance with PK analysis and Mark P. Chao at Stanford University for luciferase-expressing Raji cells. This work was supported in part by NIH grant GM95606 from the National Institute of General Medical Sciences (to JK).

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Figure 1.

Multimodality imaging of coiled-coil mediated self-assembling therapeutic system at Raji lymphoma B cell surface *in vitro* and *in vivo*. The SCID mice were intravenously injected with fluorescently labeled Raji cells *via* tail vein. Then the mice bearing Raji lymphoma were treated with the 1st-step conjugate FITC-Fab'-CCE and 2nd-step conjugate Cy5-P-(CCK)₉ consecutively. Apoptosis was induced in Raji cells by crosslinking its CD20 antigens, which was mediated by antiparallel coiled-coil formation at the cell surface. In this work, to validate assembly of two conjugates on Raji cells and crosslinking of CD20 antigens, multiple imaging studies were performed, including FMT/IVIS imaging, 3D confocal microscopy, and 4-color FACS (fluorescence-activated cell sorting). The simplified scheme is not drawn to scale. Helical wheel in the center represents CCE/CCK coiled-coil heterodimers.^[19] The view is shown looking down the superhelical axis from N-terminus of CCE and from the C-terminus of CCK. CC denotes coiled-coil peptides. E and K denote peptides in which most of the *e* and *g* position are occupied either by glutamic acid or lysine, respectively.

Figure 2. Synthesis of two fluorescently labeled conjugates FITC-Fab'-CCE (a) and Cy5-P-(CCK)₉ (b).

Figure 3.

Visualization of co-localization between FITC-Fab'-CCE and Cy5-P-(CCK)₉ on Raji cell surface. (a) Representative confocal images of Raji cells treated with FITC-Fab'-CCE and Cy5-P-(CCK)₉. The Raji lymphoma B cells were incubated with the pre-mixture of two conjugates, or consecutively exposed to FITC-Fab'-CCE and then to Cy5-P-(CCK)₉. In consecutive treatment, for blocking purpose, excess (100-fold) 1F5 antibody (Ab) was added prior to FITC-Fab'-CCE, or excess (100-fold) CCK was added prior to Cy5-P-(CCK)₉. The cells treated with Cy5-P-(CCK)₉ only (without FITC-Fab'-CCE) served as a control. FITC, green; Cy5, red. Scale bar, 20 μm. (b) z-stack confocal images of distribution patterns of lipid rafts, FITC-Fab'-CCE and Cy5-P-(CCK)₉ on the Raji cell surface. The Raji cells were consecutively treated with two conjugates. The non-treated normal Raji cells served as a control. Circular regions indicate lipid rafts clusters. Graticule size, 10 μm.

Figure 4.

“Two-step” targeting of FITC-Fab'-CCE and Cy5-P-(CCK)₉ in mice bearing NHL B-lymphoma xenografts. (a), Representative FMT images of Cy5-P-(CCK)₉ and inoculated DiR-labeled Raji cells in mice. L, liver; K, kidney. (b), Biodistribution of DiR-labeled Raji cells and Cy5-P-(CCK)₉ cells in mice. (c), Ratio of radiant efficiency, Cy5-P-(CCK)₉ to DiR Raji cells. (d), 4-color flow cytometric analysis of bone marrow cells. Day 1, nude SCID mice were intravenously injected DiR-labeled NHL B-lymphoma Raji cells *via* tail vein. Day 2, the mice bearing systemically disseminated Raji cells were randomly divided into 4 groups and received four different treatments, including Premix, Con-1h, Con-4h, and Cy5-P-(CCK)₉ only, respectively. Day 4, the mice were first scanned using FMT. Then the tissues were harvested and measured using IVIS for fluorescence signal intensity. The bone marrow cells were collected from femur and tibia, counterstained with PE anti-CD10 antibody, and analyzed by flow cytometry. The data were presented as mean standard deviation (n=3). * p<0.05.

Figure 5.

3D confocal images showed co-localization of Raji Cells, FITC-Fab'-CCE and Cy5-P-(CCK)₉ in mice tissues after intravenous administration. Day 1, SCID mice were intravenously inoculated PE-labeled Raji cells via tail vein. Day 2, the mice bearing Raji cells received intravenous injection with FITC-Fab'-CCE first, and 4 h later with Cy5-P-(CCK)₉. Day 4, the tissues, including lung, spleen, liver, and calvarium were harvested and observed under confocal microscope. PE, blue; FITC, green; Cy5, red. Graticule size is 50 μm in the images of lung, spleen and liver. In merged calvarium image, the image at UV channel was also included to provide outline of bone structure, and graticule size is 500 μm.

Figure 6.

3D confocal images of Raji Cells, FITC-Fab'-CCE and Cy5-P-(CCK)₉ in mice tissues after “Premix” treatment. Day 1, SCID mice were intravenously injected PE-labeled Raji cells via tail vein. Day 2, the mice bearing Raji cells were intravenously given the pre-mixture of FITC-Fab'-CCE and Cy5-P-(CCK)₉. Day 4, the tissues (i.e., lung, spleen, liver) were harvested and immediately imaged using confocal microscope. PE, blue; FITC, green; Cy5, red. Graticule size, 50 µm.