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# Immunogenicity of coiled-coil based drug-free macromolecular therapeutics

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ABSTRACT

A two-component CD20 (non-internalizing) receptor crosslinking system based on the biorecognition of complementary coiled-coil forming peptides was evaluated. Exposure of B cells to Fab'-peptide1 conjugate decorates the cell surface with peptide1; further exposure of the decorated cells to P-(peptide2)$_x$ (P is the *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer backbone) results in the formation of coiled-coil heterodimers at the cell surface with concomitant induction of apoptosis. The aim of this study was to determine the potential immunogenicity of this therapeutic system that does not contain low molecular weight drugs. Enantiomeric peptides (L- and D-CCE and L- and D-CCK), HPMA copolymer-peptide conjugates, and Fab' fragment-peptide conjugates were synthesized and the immunological properties of peptide conjugates evaluated *in vitro* on RAW264.7 macrophages and *in vivo* on immunocompetent BALB/c mice. HPMA copolymer did not induce immune response *in vitro* and *in vivo*. Administration of P-peptide conjugates with strong adjuvant resulted in antibody response directed to the peptide. Fab' was responsible for macrophage activation of Fab'-peptide conjugates and a major factor in the antibody induction following i.v. administration of Fab'-conjugates. There was no substantial difference in the ability of conjugates of D-peptides and conjugates of L-peptides to induce Ab response.


## 1. Introduction

Self-assembled hybrid biomaterials composed from at least two distinct classes of macromolecules are major components of smart systems with high translational potential [1]. One of the hybrid materials developed was based on graft copolymers composed from a synthetic polymer backbone and complementary peptide grafts that, when mixed, self-assemble through coiled-coil formation [2]. For example, a mixture of *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers grafted with a pair of oppositely charged coiled-coil forming peptides, CCE and CCK (graft copolymers P-CCE and P-CCK), spontaneously self-assembled into a 3D hydrogel [3,4].

Expansion of hydrogel design principles to a biological system led to the development of macromolecular therapeutics for the treatment of Non-Hodgkin lymphoma (NHL). It is well established that crosslinking of CD20 receptors at the B cell surface initiates apoptosis [5,6]. A system composed of a conjugate of Fab' fragment of anti-CD20 antibody with CCE peptide and HPMA copolymer grafted with multiple copies of the complementary CCK peptide has been designed based on this rationale. Exposure of Raji B cells to anti-CD20 Fab'-CCE conjugate decorated the cell surface with CCE (CD20 is a non-internalizing receptor) through antigen (Ag)-antibody (Ab) fragment recognition. Further exposure of the decorated cells to P-(CCK)$_x$ (P is the copolymer backbone grafted with multiple copies of CCK) resulted in the formation of CCE/CCK heterodimers at the cell surface. This second biorecognition between CCE and CCK induced the crosslinking of CD20 receptors and triggered the apoptosis of Raji B cells *in vitro* [7] and in an NHL animal model *in vivo* [8]. This is a new concept, where the biological activity of the therapeutic system is based on the biorecognition of complementary motifs. We coined the phrase "drug-free macromolecular therapeutics" for this system; no low molecular weight drug is involved, and the individual parts of the delivery system do not have apoptosis inducing activity.

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For the ultimate translation of this system into the clinics, its biocompatibility and immunocompatibility are of utmost importance [9]. There is sufficient knowledge in the literature on the biocompatibility of antibodies and antibody fragments as well as on ways to manipulate their primary structure to enhance their biocompatibility [10]. HPMA homopolymer is non-immunogenic; it does not activate lymph node cells [11] and did not induce detectable levels of antibodies in five different strains of mice following intraperitoneal administration as an alum precipitate [12]. The presence of short oligopeptide side-chains attached to polyHPMA results in a weak antibody (Ab) response. The intensity of Ab production depends on the structure of the short peptide side chain, dose, and genetic background of the mice [12]. HPMA copolymers have been used as drug carriers for decades; the biocompatibility and non-immunogenicity of HPMA copolymer-doxorubicin (adriamycin) conjugate containing a GFLG peptide spacer was determined on two inbred strains of mice [13] and validated in clinical trials (for reviews see Refs. [14–16]).

However, there is insufficient data on the potential immunogenicity of longer peptides and their conjugates with Fab' fragments and synthetic polymers. Peptides are commonly considered weak immunogens, and the production of antibodies against them requires the use of adjuvants [17]. In addition, attachment of peptides to non-immunogenic polymeric carriers results in a decrease in their immune response [18,19]. However, the response may increase upon self-assembly [20] and result in the production of conformation-specific antibodies [21,22]. Finally, the question of response to enantiomeric peptides needs to be addressed [23–25].

In this study, we have evaluated immunological properties of the drug-free macromolecular therapeutics system. To this end, we have synthesized enantiomeric peptides (L- and D-CCE and L- and D-CCK), HPMA copolymer-peptide conjugates, Fab' fragment-peptide conjugates and evaluated their immunological properties *in vitro* on RAW264.7 macrophages and *in vivo* on immunocompetent BALB/c mice. Individual samples and complementary mixtures that form coiled-coil structures were evaluated. Both, B cell and T cell responses were assessed as well as the Ab response to another Ag (ovalbumin).

## 2. Materials and methods

### 2.1. Materials

*N*-α-Fmoc protected amino acids were purchased from P3 Biosystems and AAPTEC. V-501 (4,4′-azobis(4-cyanopentanoic acid)) and V-65 (2,2′-azobis(2,4-dimethyl valeronitrile)) were purchased from Wako Chemicals (Richmond, VA). Succinimidyl-4-(*N*-maleimido-methyl)cyclohexane-1-carboxylate (SMCC) was purchased from Soltec Ventures (Beverly, MA). Chain transfer agent 4-cyanopentanoic acid dithiobenzoate (CPDB) [26] and *N*-(2-hydroxypropyl)methacrylamide (HPMA) [27] were synthesized as previously described. *N*-(3-Aminopropyl)methacrylamide (APMA) was purchased from Polysciences (Warrington, PA). 3,3′,5,5′-Tetramethylbenzidine (TMB) and all solvents were purchased from Sigma–Aldrich.

### 2.2. Synthesis of conjugates

#### 2.2.1. Polymer synthesis

A copolymer of HPMA and APMA was synthesized using reversible addition-fragmentation chain transfer (RAFT) polymerization as previously described [28]. Briefly, the monomers HPMA and APMA, chain transfer agent CPDB, and V-501 were placed in an ampule and sealed after bubbling the solution with nitrogen. The reaction proceeded at 70 °C for 18 h. After polymerization, the polymer was precipitated in acetone/ether. The copolymer end groups were modified using a 20× molar excess V-65 in methanol at 50 °C for 4 h. Pendant amine groups were converted to maleimide groups using the heterobifunctional linker SMCC [28]. Amine and maleimide contents were determined using ninhydrin and modified Ellman's assays, respectively [29,30]. The polymer molecular weight and polydispersity were determined using an ÄKTA FPLC system (GE Healthcare, Piscataway, NJ) equipped with OptilabREX and miniDAWN detectors. Superose 6 HR10/30 column (GE Healthcare) was used with a mobile phase of sodium acetate buffer and 30% acetonitrile (v/v) (pH = 6.5).

#### 2.2.2. Peptide synthesis

The peptides CCK (K VSALKEK VSALKEE VSANKEK VSALKEK VSALKE) and CCE (E VSALEKE VSALEKK NSALEKE VSALEKE VSALEK) [28] were synthesized (Fig. 1) using solid phase synthesis on a PS3™ peptide synthesizer (Protein Technologies, Tucson, AZ). The N terminus was optionally functionalized with a spacer of CYGG (denoted as "sh") or SMCC capped YGG spacer (denoted as "mal"). In the case of L-CCEmal, the N terminus was functionalized with 3-maleimidopropionic acid instead of SMCC. Peptides of L- and D-chirality were synthesized. In total, eight different peptides were synthesized (L-CCKsh, D-CCKsh, L-CCEsh, D-CCEsh, L-CCKmal, D-CCKmal, L-CCEmal, D-CCEmal). All peptides were purified using reverse phase high-performance liquid chromatography (RP-HPLC, Agilent Technologies 1100 series), and the molecular weights were confirmed using MALDI-TOF mass spectrometry (UltrafleXtreme, Bruker Daltonics). The mass spectra of peptides are in Supplementary Data Figs. S1–S8.

#### 2.2.3. Polymer conjugate synthesis

Peptides were 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 (Fig. 2). The polymer and peptide were dissolved in PBS with 10 mM tris(2-carboxyethyl)phosphine (TCEP). Attachment was allowed to proceed overnight. Unconjugated peptides were removed using ultrafiltration. The peptide content on the graft copolymers was determined using amino acid analysis. Four different graft copolymers were produced: P-L-CCK, P-D-CCK, P-L-CCE, P-D-CCE. The composition of the conjugates is in Table 1; a representative size exclusion chromatogram is on Fig. S9 and CD spectra on Figs. S10 and S11.

#### 2.2.4. Fab' and Fab'-peptide conjugates preparation

Fab' fragment was prepared as previously described [28]. Briefly, 1F5 monoclonal Ab was prepared by culturing a hybridoma cell line in a CellMax bioreactor (Spectrum Laboratories, Rancho Dominguez, CA). Ab was harvested from the reactor and purified on a protein G column. Purified 1F5 Ab was then digested using pepsin (10 w%) in citric buffer (pH 4) for 2 h at 37 °C. $F(ab')_2$ was isolated using ultrafiltration to remove the digest products. $F(ab')_2$ was reduced using 10 mM TCEP in PBS for 1 h at 37 °C. TCEP was removed using ultrafiltration. Maleimide functionalized peptides were added to the Fab' solution in 20× molar excess. Unconjugated peptides were removed using ultrafiltration. The 1F5, $F(ab')_2$, Fab' and Fab'-peptide conjugates were analyzed using FPLC (Fig. S12) and SDS-PAGE (Fig. S13). The following Fab'-conjugates were prepared: Fab'-L-CCE, Fab'-L-CCK, Fab'-D-CCE, Fab'-D-CCK.

### 2.3. Cells

Mouse macrophage cell line RAW264.7 was maintained in complete Dulbecco's modified Eagle's medium (D-MEM; Sigma–Aldrich) supplemented with 10% fetal bovine serum (Hyclone, Thermo Fisher Scientific), 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 100 U/ml penicillin and 100 μg/mL streptomycin (Gibco/Life

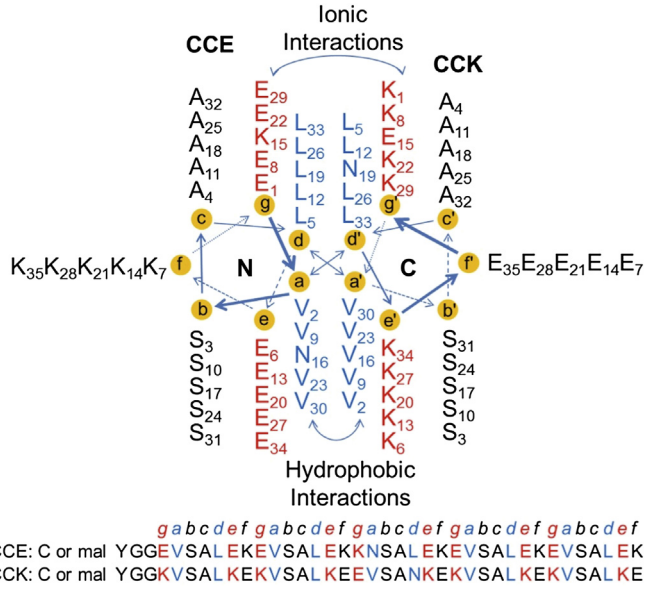


**Fig. 1.** Helical wheel diagram of the anti-parallel heterodimer of CCE/CCK [4]. The heptad repeats are labeled a-f for CCE and a'-f' for CCK. All peptides were modified with a YGG spacer and were functionalized with either cysteine or a heterobifunctional linker bearing a reactive maleimide group.