Biorecognition

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Drug-Free Macromolecular Therapeutics: Induction of Apoptosis by Coiled-Coil-Mediated Cross-Linking of Antigens on the Cell Surface**

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Molecular biorecognition is at the center of all biological processes and forms the basis for the design of precisely defined smart systems, including targeted therapeutics, imaging agents, biosensors, and stimuli-sensitive and self-assembled biomaterials. The self-assembly of hybrid materials composed of synthetic and biological macromolecules is mediated by the biorecognition of biological motifs.[1] We have previously designed self-assembling hybrid hydrogel systems composed of a synthetic *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer backbone and coiled-coil peptide motifs; our results showed that it is possible to impose properties of a well-defined coiled-coil peptide on a whole hybrid hydrogel.[2] Recently, we designed a pair of oppositely charged pentaheptad peptides (CCE and CCK) that formed antiparallel coiled-coil heterodimers and served as physical cross-linkers.[3a] HPMA graft copolymers CCE–P and CCK–P (P is the HPMA copolymer backbone) self-assembled into hybrid hydrogels with a high degree of biorecognition.[3]

We hypothesized that this unique biorecognition of CCK and CCE peptide motifs could be extended beyond biomaterials design and applied to a living system to mediate a biological process. This approach would provide a bridge between the design of biomaterials and the design of macromolecular therapeutics.

To verify this hypothesis, we chose to study the induction of apoptosis in CD20-positive cells. CD20 is one of the most reliable biomarkers for B-cell non-Hodgkin lymphoma (NHL).[4a,b] It functions as a cell-cycle-regulatory protein[4c] that either controls or functions as a store-operated calcium channel. CD20 also forms dimers and tetramers[4d] constitutively associated with lipid rafts of the cell membrane.[4b] It is a noninternalizing antigen that remains on the cell surface when bound to a complementary antibody (Ab).[4e] However, the cross-linking of CD20-bound antibodies with a secondary antibody results in apoptosis.[4f] To exploit this phenomenon, we designed a system composed of CCE and CCK peptides, the Fab′ fragment of the 1F5 anti-CD20 antibody, and HPMA copolymer (Figure 1). The exposure of CD20+ Raji B cells to Fab′–CCE resulted in the decoration of the cell surface with multiple copies of the CCE peptide through antigen–antibody-fragment biorecognition. Further exposure of the decorated cells to HPMA copolymer grafted with multiple copies of CCK resulted in the formation of CCE–CCK coiled-coil heterodimers on the cell surface. This second biorecog-

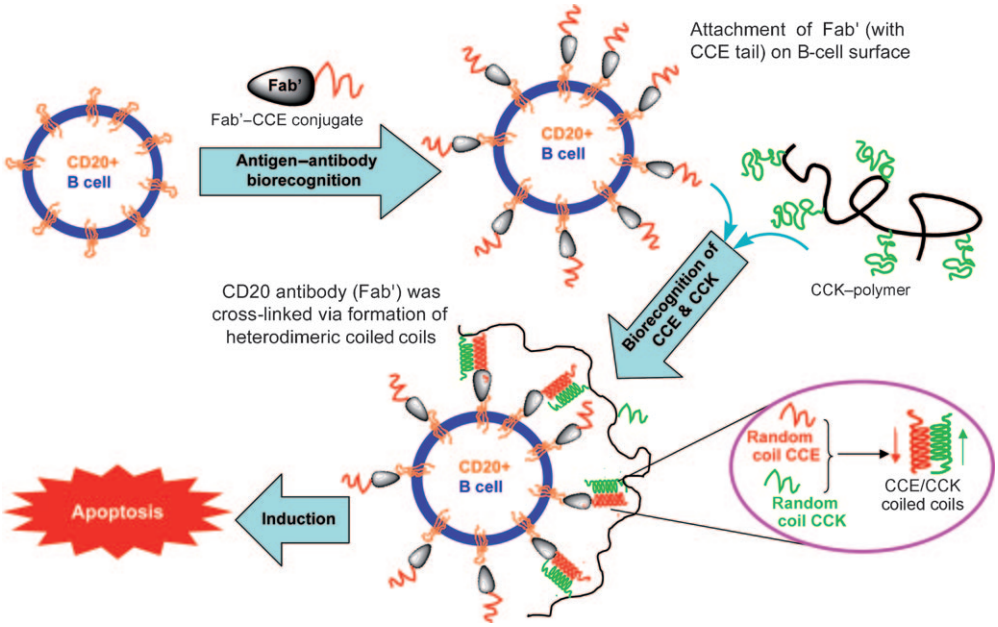

Figure 1. Induction of apoptosis in human Burkitt's NHL Raji B cells by cross-linking of its CD20 antigens mediated by antiparallel coiled-coil formation at the cell surface. The simplified schematic diagram is not drawn to scale.

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nition event induced the cross-linking of CD20 receptors and triggered the apoptosis of Raji B cells.

The peptides CCE and CCK were prepared by solid-phase peptide synthesis.[3a] Their purity and identity were confirmed by reversed-phase HPLC and MALDI-TOF mass spectrometry (see Figures S1 and S2 in the Supporting Information).

The HPMA graft copolymer CCK–P was synthesized in a three-step process (Figure 2a; for details, see the Supporting Information).[3a] First, HPMA was copolymerized with *N*-(3-aminopropyl)methacrylamide, and optionally with *N*-methacryloylaminopropyl fluorescein thiourea through free-radical polymerization. The amino groups at the side-chain

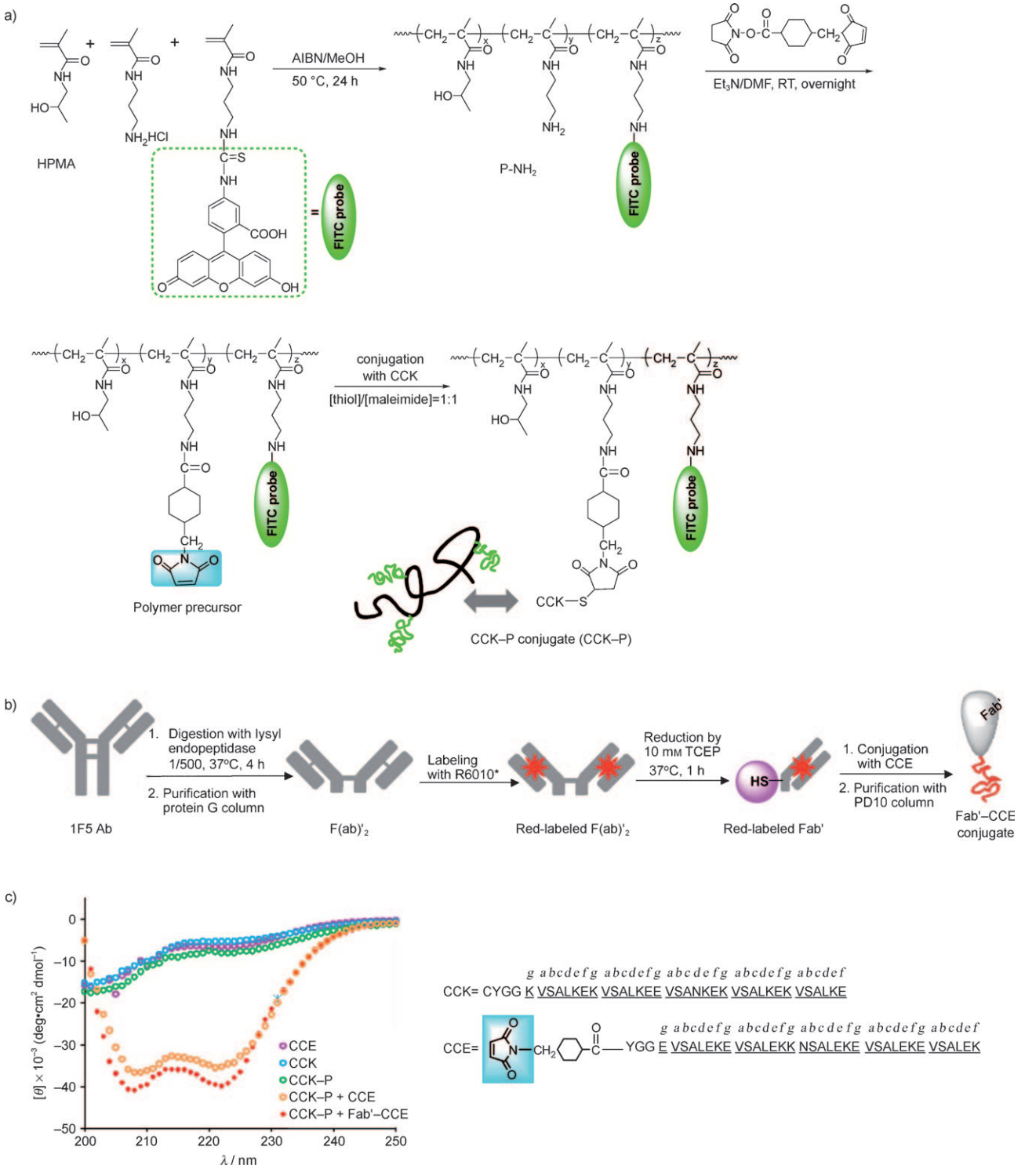

Figure 2. a) Synthesis of $(CCK)_9$–P. b) Synthesis of Fab′–$(CCE)_1$. c) CD spectra of CCE, CCK, $(CCK)_9$–P, and equimolar mixtures of CCE and $(CCK)_9$–P, and Fab′–$(CCE)_1$ and $(CCK)_9$–P. [CCE] = [CCK] = [Fab′–$(CCE)_1$] = 50 μM; [$(CCK)_9$–P] = 5.60 μM. Data were acquired at 25 °C in PBS (pH 7.4). AIBN = azobisisobutyronitrile, DMF = *N*,*N*-dimethylformamide, FITC = fluorescein isothiocyanate, R6010 = rhodamine Red-X succinimidyl ester.

termini were then converted into maleimido groups by treatment with succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate. Finally, CCK was attached to the copolymer by thiol–maleimide chemistry through the N-terminal cysteine residue of CCK. The number-average molecular weight, M_n, of the copolymer P was 99.6 kDa, and the average number of CCK grafts per macromolecule was 8.94 (the graft copolymer is abbreviated as $(CCK)_9$–P). The murine 1F5 anti-CD20 IgG2a antibody[5] was prepared from the anti-CD20 hybridoma clone 1F5 (ATCC, Bethesda, MD) in a CellMax bioreactor (see the Supporting Information) and enzymatically digested with lysyl endopeptidase. The $F(ab')_2$ fragment was isolated on a protein G column.[6] After labeling with Rhodamine Red-X, $F(ab')_2$ was reduced by tris(2-carboxyethyl)phosphane (TCEP) and then conjugated with CCE to produce the Rhodamine Red-X labeled Fab′–CCE conjugate, Fab′–$(CCE)_1$ (Figure 2b; see also Figure S3 in the Supporting Information).

The biorecognition of Fab′–$(CCE)_1$ and $(CCK)_9$–P was first evaluated by circular dichroism (CD) spectrometry. A pronounced coiled-coil signal (minima at 208 and 222 nm) was observed upon the mixing of CCE/Fab′–$(CCE)_1$ and $(CCK)_9$–P (Figure 2c). Dynamic light scattering (DLS) measurements revealed that within 1 hour after mixing, the effective diameter of particles in the equimolar mixture of Fab′–$(CCE)_1$ and $(CCK)_9$–P increased significantly (see Figure S4 in the Supporting Information).

Human Burkitt's NHL Raji B cells were used to demonstrate the biorecognition of coiled coils at the cell surface and apoptosis induction as a result of CD20 cross-linking. They were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2 (v/v). The exposure of Raji B cells to Fab′–$(CCE)_1$ led to decoration of the cell surface with the CCE peptide, as shown by confocal fluorescence microscopy (Figure 3a). In contrast, the exposure of Raji B cells to $(CCK)_9$–P did not result in detectable deposition of the graft copolymer at the cell surface under the experimental conditions used (Figure 3b). The exposure of Raji B cells to a high-avidity multivalent construct prepared by the premixing of Fab′–$(CCE)_1$ and $(CCK)_9$–P resulted in excellent binding to the cell surface (Figure 3c_1–c_3). This result is in agreement with our studies on the interaction of CD20 with multivalent HPMA copolymer–Fab′ conjugates.[6b] Finally, the exposure of Raji B cells predecorated with the CCE peptide to $(CCK)_9$–P (consecutive exposure) resulted in the attachment of $(CCK)_9$–P to the cell surface (Figure 3d_1–d_3). This result suggests that coiled-coil heterodimers were formed at the cell surface with the concomitant cross-linking of CD20 receptors. It demonstrates outstanding biorecognition between CCE bound to the cell surface and CCK attached as grafts to the HPMA copolymer. Apparently, the proteins present in FBS did not interfere with the antiparallel heterodimerization. For details on the biorecognition studies, more images, and additional controls, see the Supporting Information (Figures S5–S7).

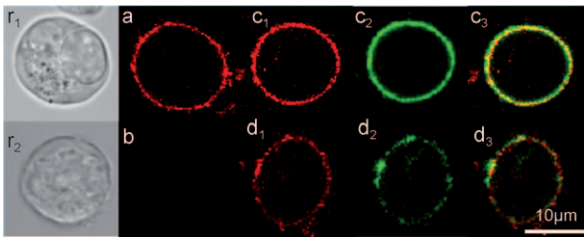

Figure 3. Biorecognition of Fab′–CCE and CCK–P on the surface of Raji B cells (r_1, r_2: reference cells under transmitted light). a) The exposure of cells to Fab′–$(CCE)_1$ (0.5 μM, labeled with Rhodamine Red-X) resulted in decoration of the cell surface with CCE. b) The exposure of cells to $(CCK)_9$–P ([CCK] = 25 μM, labeled with FITC) did not result in staining. c_1–c_3) Exposure of cells to premixed Fab′–$(CCE)_1$ (0.5 μM) and $(CCK)_9$–P ([CCK] = 25 μM). d_1–d_3) Consecutive exposure of cells to Fab′–$(CCE)_1$ (0.5 μM) followed (1 h later) by $(CCK)_9$–P ([CCK] = 25 μM). a, c_1, and d_1: red channel for Rhodamine Red-X; b, c_2, and d_2: green channel for FITC; c_3 and d_3: overlay of red and green channels. Images of individual cells are shown.

Three assays were used to demonstrate apoptosis induction following the cross-linking of CD20 antigens: a caspase 3 activity assay, an annexin V/propidium iodide assay, and a TUNEL (terminal deoxynucleotidyl transferase dUTP (deoxyuridine triphosphate) nick end labeling) assay. The caspase 3 assay was used to evaluate the time dependence of apoptosis induction (Figure 4). The exposure of cells to the individual components Fab′–$(CCE)_1$ or $(CCK)_9$–P resulted in a very low percentage of cell death, independent of the incubation interval. However, a time dependency was observed for the coiled-coil-based apoptosis-induction systems: treatment with a mixture of Fab′–$(CCE)_1$ and $(CCK)_9$–P, and the consecutive exposure of cells to Fab′–$(CCE)_1$ followed by $(CCK)_9$–P. The highest levels of apoptosis after incubation for 6 hours were observed with premixed Fab′–

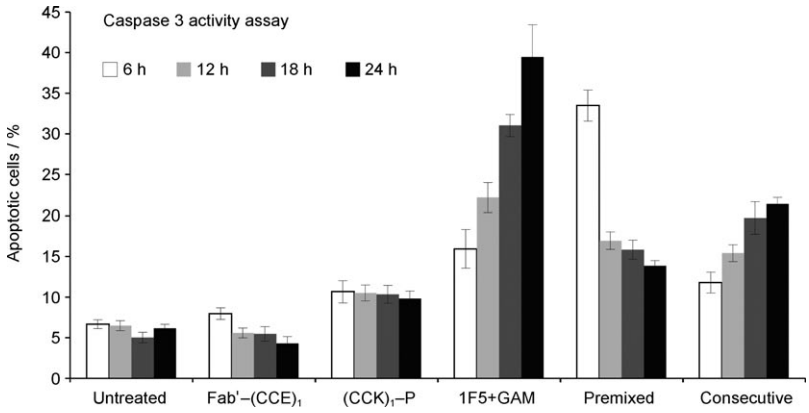

Figure 4. Time dependence of apoptosis induction in Raji B cells. Untreated: untreated cells; Fab′–$(CCE)_1$: single-component control at 0.5 μM; $(CCK)_9$–P: single-component control ([CCK] = 25 μM); 1F5 + GAM: addition of 1F5 Ab (0.2 μM) followed (1 h later) by the secondary antibody GAM (10 μg mL^{-1}); premixed: premixture of Fab′–$(CCE)_1$ (0.5 μM) and $(CCK)_9$–P ([CCK] = 25 μM); consecutive: consecutive addition of Fab′–$(CCE)_1$ (0.5 μM) followed (1 h later) by $(CCK)_9$–P ([CCK] = 25 μM). Results are presented as mean values ± standard deviation ($n = 3$).

$(CCE)_1$ and $(CCK)_9$–P, whereas for both consecutive-addition systems, Fab′–$(CCE)_1$ + $(CCK)_9$–P and the control 1F5 + goat antimouse (GAM) secondary antibody, the extent of apoptosis increased with increasing incubation time, with the highest level observed after 24 hours. These results probably reflect different levels of destabilization of the plasma-membrane integrity following exposure to a multivalent high-avidity conjugate versus a monovalent Fab′–$(CCE)_1$ conjugate.

In further experiments, the systems were compared at the time intervals corresponding to maximum apoptosis, that is, 6 hours for premixture and 24 hours for consecutive addition. The extent of apoptosis could be increased by manipulating the concentration of the components. An increase in the Fab′–$(CCE)_1$ concentration from 0.5 to 1 μM led to an increase in the amount of apoptotic cells from 21 to 32% in the clinically relevant consecutive-addition system (Figure 5a).

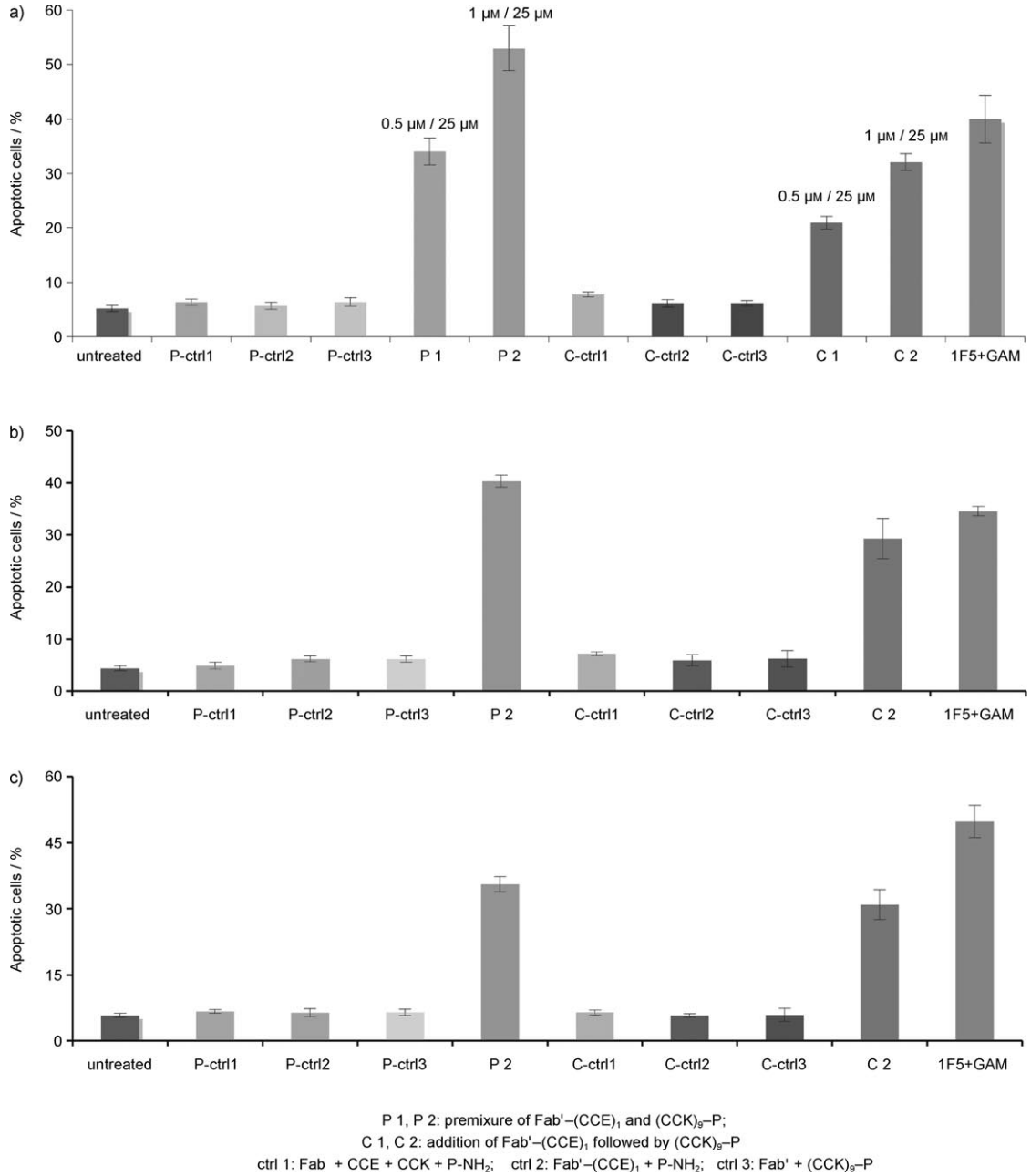

Figure 5. Coiled-coil-mediated induction of apoptosis of Raji B cells, as assessed by: a) caspase 3 activity, b) an annexin V/propidium iodide assay, and c) a TUNEL assay. The concentrations of 1F5 and GAM were 0.2 μM and 10 $\mu g\,mL^{-1}$, respectively. The concentrations of Fab′–$(CCE)_1$ and CCK in $(CCK)_9$–P were 0.5 and 25 μM, respectively, for P 1 and C 1 in the caspase 3 assay, and 1 and 25 μM, respectively, for all other experiments. P-ctrl1: a premixture of Fab′ (1 μM), CCE (1 μM), CCK (25 μM), and P-NH_2 (2.80 μM); P-ctrl2: a premixture of Fab′–$(CCE)_1$ (1 μM) and P-NH_2 (2.80 μM); P-ctrl3: a premixture of Fab′ (1 μM) and $(CCK)_9$–P (2.80 μM); C-ctrl1: Fab′ (1 μM) and CCE (1 μM) for 1 h, and then CCK (25 μM) and P-NH_2 (2.80 μM); C-ctrl2: Fab′–$(CCE)_1$ (1 μM) for 1 h, then P-NH_2 (2.80 μM); C-ctrl3: Fab′ (1 μM) for 1 h, then $(CCK)_9$–P ([CCK] = 25 μM/[P-NH_2] = 2.80 μM). Results are presented as mean values ± standard deviation ($n = 3$).

The results of annexin V (Figure 5b) and TUNEL (Figure 5c) assays corroborated the efficacy of the system in the presence of FBS. Comparable levels of apoptosis induction (ca. 30%) were observed for consecutively added Fab′–$(CCE)_1$ (1 μM) and $(CCK)_9$–P ([CCK] = 25 μM), consecutively added 1F5 and GAM (ca. 40%), and premixed Fab′–$(CCE)_1$ and $(CCK)_9$–P (ca. 40%). The apoptotic levels observed in our experiments are comparable to those found upon the exposure of Raji B cells to multivalent antibody conjugates: HPMA copolymers containing multiple Fab′ fragments,[6b] dextran–antibody (rituximab) conjugates,[7a] and rituximab dimers.[7b]

Control experiments validated the hypothesis that the coiled-coil heterodimerization of CCE with CCK with concomitant cross-linking of the CD20 antigen is responsible for apoptosis induction. The levels of apoptosis observed after the exposure of Raji B cells to: a) a mixture of Fab′, CCE, CCK, and P-NH_2; b) a mixture of Fab′-$(CCE)_1$ and P-NH_2; and c) a mixture of Fab′ and $(CCK)_9$-P were very low, and similar to those observed for untreated cells (Figure 5a–c, controls 1, 2, and 3).

Further optimization of the system might result in even higher apoptosis levels. The factors to be studied include: optimization of the concentration and the timing of consecutive addition of the components, the use of D-amino acid sequences, the design of shorter sequences, switching of the peptides (evaluation of Fab′–CCK + CCE–P), and the insertion of a spacer between Fab′ and the peptide.

Several factors contributed to the successful design of the new drug-free therapeutic system: a) the use of the 1F5 antibody, the binding of which to CD20+ cells does not induce apoptosis: a secondary GAM antibody[8] is needed; b) the design of the CCE and CCK sequences[3a] (see Figure S1 in the Supporting Information): antiparallel heterodimer formation reduces the steric hindrance of the polymer chain during the binding of $(CCK)_9$–P to Raji cells decorated with CCE motifs and enhances the probability of "in-register" alignment of the CCE–CCK heterodimer; c) the HPMA copolymer employed for the conjugation of multiple copies of one of the peptides (CCK): HPMA copolymers have been widely used in drug-delivery systems;[9] their biocompatibility has been proven in animal models[10a–c] and in clinical trials;[10d] d) the choice of the CD20 antigen: CD20 is expressed on most NHL malignant cells as well as on normal B cells; however, it is not expressed on stem cells and mature plasma cells; consequently, normal numbers of B cells can be restored after treatment.[11]

We have presented a new approach to apoptosis induction mediated by the biorecognition of coiled-coil-forming peptide segments on the cell surface. The fact that biorecognition of coiled-coils at the cell surface occurred in media containing 10% FBS indicates the specificity of the CCE–CCK interaction and bodes well for future in vivo experiments and for the development of efficient drug-free macromolecular therapeutics. The important feature of this design is the absence of low-molecular-weight cytotoxic compounds. The concept of drug-free macromolecular therapeutics could be expanded by using different components in the design. For example, the Fab′ fragment could be replaced by antigen-binding saccharides[12a] or by peptides[12b,c] selected by combinatorial methods.

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