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Polymer Therapeutics with a Coiled Coil Motif Targeted against Murine BCL1 Leukemia

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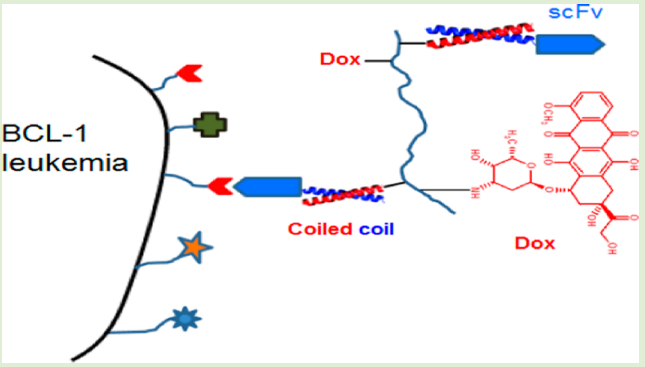
ABSTRACT: The specificity of polymer conjugates based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) bearing cytostatic drugs for cancer cells could be significantly increased by the incorporation of a suitable targeting ligand, such as a monoclonal antibody (mAb). However, direct binding of the protein to the polymer carrier could cause considerable problems, such as decreasing the binding capacity of mAb to its target. Here, we introduce a novel strategy of joining a targeting moiety to a polymeric conjugate with cytostatic drug. The scFv of B1 mAb (specific for BCL1 leukemia cells) was tagged with peptide K ($(VAALKEK)_4$). Peptide E ($(VAALEKE)_4$), which forms a stable coiled coil structure heterodimer with peptide K, was assembled with the HPMA copolymers bearing doxorubicin. Such targeted polymeric conjugates possess very selective and high binding activity toward BCL1 cells. Similarly, targeted polymeric conjugates exert approximately 100 times higher cytostatic activity toward BCL1 cells in comparison to nontargeted conjugates *in vitro*. At the same time, the conjugates have comparable and rather low cytostatic activity for 38C13 cells, which are used as a negative control, *in vitro*.



INTRODUCTION

Polymer drug conjugates that are targeted using monoclonal antibodies belong to the most advanced category of nanomedicines in the field of tumor therapy.[1] Unfortunately, the covalent attachment of an antibody (or generally any protein/glycoprotein molecule) to a polymer carrier is accompanied by several significant problems. First, modification of the protein with a multivalent reactive polymer precursor rarely leads to a well-defined product. Second, the biological activity of the protein is very likely altered after such modification. Consequently, eventual regulatory approval of the resulting heterogeneous polymer−protein conjugates becomes a formidable and complicated task for any pharmaceutical company trying to get their product through clinical trials. Hence, the demand for a new technology that is suitable for the preparation of site-specific polymer−protein conjugates is quite urgent.

Recently, we described[2] a new method of conjugating two macromolecules based on a strong and specific interaction between two peptides to form a heterodimeric coiled coil structure. A similar approach based on the coiled coil heterodimers was described also by several other investigators in the field of biomedicinal polymers.[3−9]

We have designed and characterized synthetic hydrophilic copolymers based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) bearing multiple peptide sequences $(VAALEKE)_4$, called peptide E, attached to the polymer backbone via a copper-catalyzed azide−alkyne cycloaddition, "click" chemistry.[10] The copolymer formed a stable noncovalent complex with a recombinant single-chain fragment (scFv) of antibody M75 containing at the C-terminus the sequence $(VAALKEK)_4$, which is called peptide K. The formation of the complex was mediated by a strong heterodimeric interaction between peptides E and K, which form a coiled coil structure. This noncovalent association of peptides represents a rapid, stable and well-characterized binding of the targeting ligand to the polymer conjugate. The antibody M75 specifically binds to carbonic anhydrase IX (CA IX), a transmembrane protein overexpressed in a wide variety of tumor cell types.[11] The specific binding of the polymer−scFv complex to CA IX was confirmed by ELISA.

In this paper, we introduce a more advanced system intended for affinity therapy with a polymer-bound cytostatic drug

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consisting of HPMA copolymers with the antitumor drug doxorubicin (Dox) and the recombinant scFv of B1 mAb, which recognizes the idiotype of surface IgM expressed on mouse BCL1 leukemia cells. The B1 mAb very specifically binds to murine BCL1 leukemia cells; thus, they have high potential to be used as a targeting moiety in the polymer−Dox conjugates designed for selective leukemia-cell-targeted therapy. The major aim of this paper was to verify the binding activity of the B1 mAb-targeted polymer conjugates to BCL1 cells and the selectivity of such binding using a 38C13 cell line (a negative control), which is a mouse B-cell lymphoma closely resembling BCL1 cells. These cells also express surface IgM but with a different epitope not recognized by the B1 mAb. Verification of the effect of the specificity of such targeting to BCL1 cell receptors on the accumulation of doxorubicin in the cells and the determination of the cytostatic activity of the B1 mAb-targeted polymeric conjugates and their specificity *in vitro* are important aims of this study. Nontargeted polymer conjugates with peptide E-bearing doxorubicin and HPMA copolymer-bound doxorubicin (PK1) were selected as negative controls to evaluate the improvement of the cell-specific cytostatic activity. The 38C13 cells were used as a receptor negative control to verify the specificity of the cytostatic activity of the targeted conjugates.

The major advantage of the described "coiled coil" self-assembly method over standard covalent modification of antibodies with polymers is the well-defined structure of the complex. The presented drug delivery system is also ideal for bivalent (or multivalent) targeting. The bivalent targeting of polymer therapeutics is one of the topics of our current investigations. Moreover, in situ self-assembly of independently stored polymer-drug and targeting antibody enabling preparation of the conjugate "on request" for personalized therapy could be another advantage of the described system in future.

EXPERIMENTAL SECTION

Chemicals. 2,2′-Azobis(isobutyronitrile) (AIBN), *N*,*N*′-dicyclohexylcarbodiimide (DCC), dichloromethane (DCM), 4-(dimethylamino)pyridine (DMAP), dimethyl sulfoxide (DMSO), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), 1-hydroxybenzotriazole (HOBt), *N*-hydroxysuccinimide (HOSu), pentamethylcyclopentadienyl(cyclooctadiene)ruthenium(II) chloride (Ru(COD)), triphenylchloromethane (TrtCl), and thiazolidine-2-thione (H-TT) were purchased from Sigma-Aldrich, Czech Republic.

Ethyldiisopropylamine (DIPEA), *N*,*N*-dimethylformamide (DMF), 9-fluorenylmethoxycarbonyl-amino acids (Fmoc-aa), and (benzotriazol-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate (PyBOP) were purchased from Iris Biotech, GmbH, Germany. *N*-[2-(2-[2-(2-Azidoethoxy)ethoxy]ethoxy)ethyl]biotin amide (azide-Peg_4-biotin) was purchased from Click Chemistry Tools, Scottsdale, AZ.

All other solvents and reagents were purchased from Sigma-Aldrich, Czech Republic. All chemicals and solvents were of analytical grade. Solvents were purified and dried using standard procedures.

Methods. The homogeneity of the peptide derivatives and the progress of the conjugation reactions were monitored by reversed-phase HPLC using Chromolith Performance RP-18e columns, 100 × 4.6 mm (Merck, Germany), with a linear gradient of water−acetonitrile (0−100% acetonitrile) in the presence of 0.1% TFA with a UV−vis diode array detector (Shimadzu, Japan). The amino acid analysis of the hydrolyzed samples (6 M HCl, 115 °C, 18 h in a sealed ampule) was performed on a reversed-phase Chromolith Performance RP-18e column, 100 × 4.6 mm (Merck, Germany), using precolumn derivatization with phthalaldehyde (OPA) and 3-sulfanylpropanoic acid (excitation at 229 nm, emission at 450 nm) and a gradient elution of 0−100% solvent B over 18 min at a flow rate of 1.0 mL/min (solvent A, 0.05 M sodium acetate buffer, pH 6.5; solvent B, 300 mL of 0.17 M sodium acetate and 700 mL of methanol). The molecular mass of the peptide products was determined using mass spectrometry performed on an LCQ Fleet mass analyzer with electrospray ionization (ESI MS) (Thermo Fisher Scientific, Inc., Waltham, MA). The determination of the molecular weights and polydispersity of the copolymers was carried out by size exclusion chromatography (SEC) on a Shimadzu HPLC system (Shimadzu) equipped with refractive index, UV, and multiangle light scattering (LS) DAWN 8 EOS (Wyatt Technology Corp., Santa Barbara, CA) detectors using either a Superose 6 column (Pharmacia) (0.3 M acetate buffer, pH 6.5) or a TSK 3000 SW_{XL} column (Tosoh Bioscience, Japan) (50% methanol, 0.1% TFA) at a flow rate of 0.5 mL/min. The calculation of molecular weights from the light-scattering intensity was based on the known injected mass, assuming 100% mass recovery. The content of thiazolidine-2-thione (TT) groups and Dox was determined spectrophotometrically on a Helios Alpha UV/vis spectrophotometer (Thermospectronic, U.K.) using the absorption coefficients for TT in DMSO (ε_{306} = 10 280 L·mol^{-1}·cm^{-1}) and for Dox in methanol (ε_{488} = 9900 L·mol^{-1}·cm^{-1}). Solid-phase peptide synthesis and solid-phase fragment condensation of protected peptide fragments were performed on an AVSP-2 multiple automatic peptide synthesizer (Development Workshops of the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic).

Azide Derivative of Peptide E (1). The linear coiled coil peptide consisting of four repeating heptads terminating with an azide group was assembled using solid phase fragment condensation of fully protected heptapeptides, as described earlier.[2]

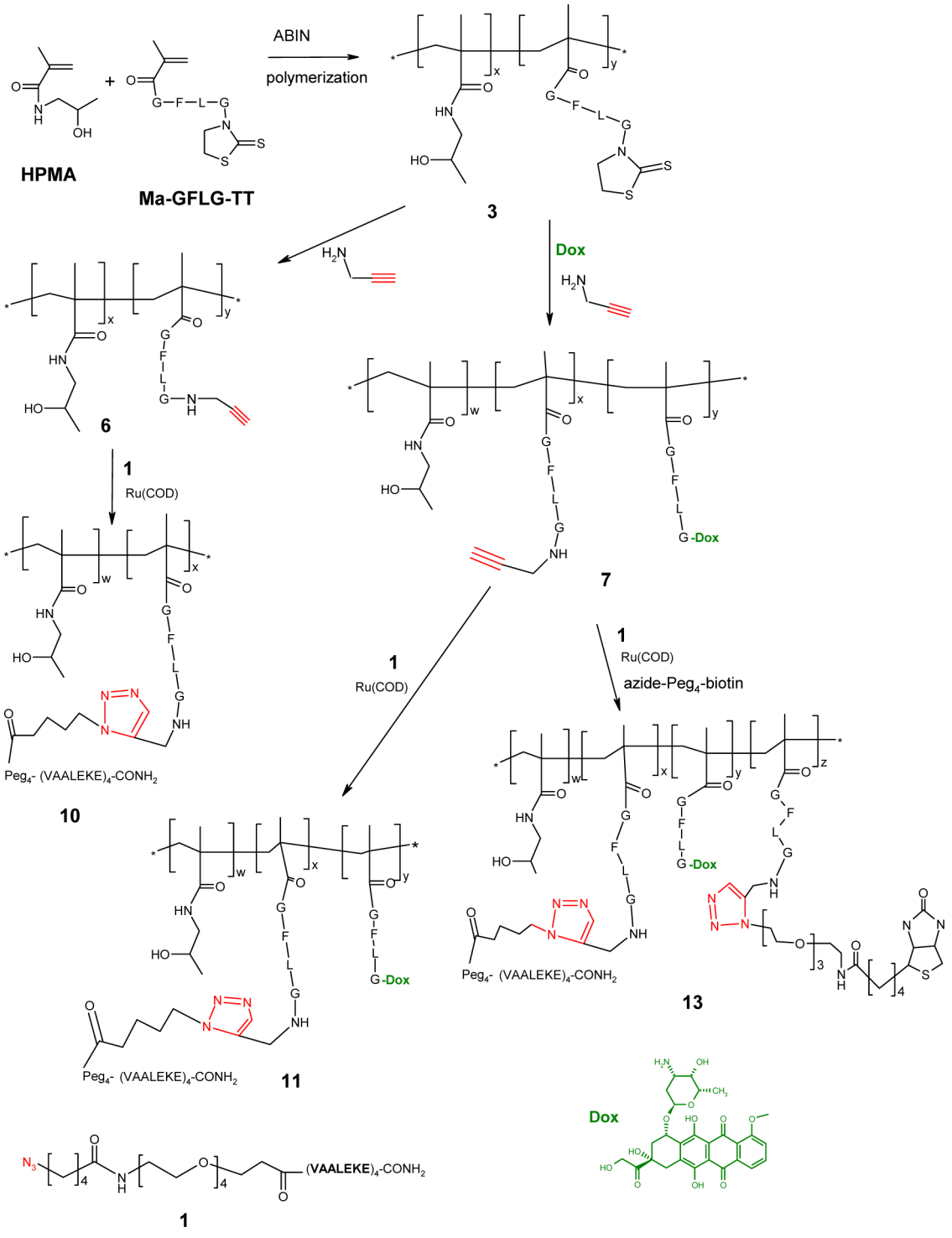
Trt-GFLG-OH. The Fmoc protecting group was removed from 1 g of 4-Fmoc-hydrazinobenzoyl AM Novagel resin with a piperidine−DMF solution (1:4). The loading of the resin was determined by spectrophotometric measurement of the Fmoc group (0.46 mmol/g). The linear tetrapeptide Gly-Phe-Leu-Gly was assembled using a solid-phase synthesis starting from the C-terminus using standard Fmoc procedures, including the consecutive addition of the *N*-Fmoc-protected amino acid (3 equiv), PyBOP (3 equiv), HOBt (3 equiv), and DIPEA (6 equiv) in DMF. Fmoc groups were removed using piperidine−DMF (1:4). After removal of last Fmoc group, the N-terminus of the peptide was reacted with TrtCl (3 equiv) in the presence of DIPEA (3 equiv) in DCM for 4 h. The peptide was cleaved from the resin (350 mg, 0.16 mmol of peptide) with a 0.05 M aqueous solution of Cu (II) acetate (16 mg, 0.08 mmol), pyridine (130 μL, 1.6 μmol) and dioxane (5.25 mL), while the resin was bubbled with air vigorously for 4 h. The resin was removed by filtration and washed with DCM. The combined filtrates were washed with a 1 M aqueous solution $KHSO_4$ and water; the organic phase was dried with anhydrous Na_2SO_4 and evaporated to dryness, yielding 28 mg (28%) of white solid. The product was characterized by reversed-phase HPLC and ESI MS (calculated 634.3; found 633.2, M − H).

H_2N-GFLG-Dox (2). H_2N-GFLG-Dox was prepared as described earlier.[12] The yield was 125 mg (68%) of red solid **2**. The product was characterized by reversed-phase HPLC and ESI−MS (calculated 918.0; found 916.8, M − H).

Synthesis of Monomers. *N*-(2-Hydroxypropyl)methacrylamide (HPMA) was prepared by the reaction of methacryloyl chloride with 1-aminopropan-2-ol in DCM.[13] *N*-Methacryloylglycylglycine (Ma-GG-OH) was prepared by the Schotten−Baumann acylation of glycylglycine with methacryloyl chloride in an aqueous alkaline medium. 3-(*N*-Methacryloylglycylglycyl)thiazolidine-2-thione (Ma-GG-TT) was prepared by the reaction of Ma-GG-OH with H-TT in DMF in the presence of *N*,*N*′-dicyclohexylcarbodiimide.[14] *N*-Methacryloyl glycylphenylalanylleucylglycine (MA-GFLG-OH) was assembled by automatic solid phase peptide synthesis on 2-chlorotrityl chloride resin starting from the C-terminus using standard Fmoc procedures, including the consecutive addition of the N-Fmoc-protected amino acid (2.5 equiv), PyBOP (2.5 equiv), HOBt (2.5 equiv), and DIPEA (5.0 equiv) in DMF.

The Fmoc groups were removed using piperidine−DMF (1:4). After removal of the last Fmoc group, the methacrylic acid (2.5 equiv)

Scheme 1. Synthesis of Polymer–Peptide Conjugates Prepared by Free Radical Polymerization



was attached to the N-terminus of the tetrapeptide in the presence of PyBOP (2.5 equiv), HOBt (2.5 equiv), and DIPEA (5.0 equiv) in DMF. Cleavage of the unprotected monomer from the resin was performed using a solution of 30% HFIP in DCM (20 mL/g of resin) in the presence of 4-(1,1,3,3-tetramethylbutyl)pyrocatechol as a polymerization inhibitor for 2 h. The resin was filtered off and rinsed with TFA. The filtrate was concentrated under vacuum and precipitated with diethyl ether. The precipitate was isolated by filtration and dried in vacuum.

3-(*N*-Methacryloylglycyl-phenylalanylleucylglycyl)thiazolidine-2-thione (Ma-GFLG-TT) was synthesized by the reaction of Ma-GFLG-OH with 4,5-dihydrothiazole-2-thiol in the presence of DCC and DMAP.[14]

Reactive Copolymers with TT Groups. The copolymer poly(HPMA-*co*-Ma-GFLG-TT) (**3**) was prepared by solution radical copolymerization of HPMA (87.5 mol %) and Ma-GFLG-TT (12.5 mol %) in DMSO at 60 °C for 6 h (Scheme 1). The concentration of comonomers in the polymerization mixture was 13% (w/w), and that of AIBN was 2% (w/w).[14]

The copolymer poly(HPMA-*co*-Ma-GG-TT) (**4**) was prepared by reversible addition–fragmentation chain transfer (RAFT) polymerization of HPMA (90 mol %, 200 mg) and Ma-GG-TT (10 mol %, 47

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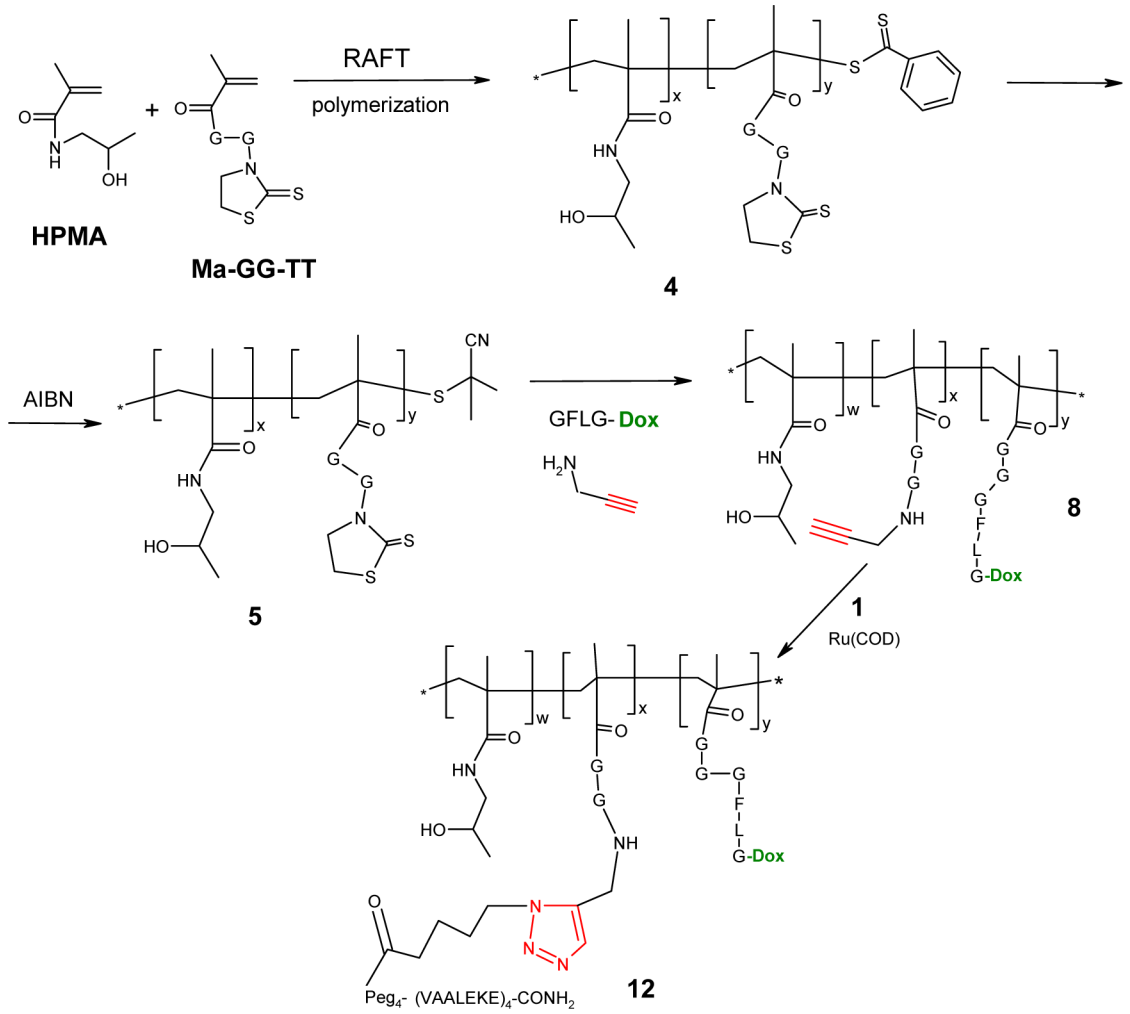
Scheme 2. Synthesis of Polymer–Peptide Conjugates Prepared by RAFT Polymerization



mg) using AIBN (0.85 mg) as an initiator and 4-cyano-4-thiobenzoylsulfanylpentanoic acid (2.29 mg) as a chain transfer agent. The polymerization mixture was dissolved in *tert*-butyl alcohol (1.724 mL, 0.9 M solution of monomers), transferred into a glass ampule, bubbled with Ar and sealed. After 6 h at 70 °C, the product was isolated by precipitation with acetone; the precipitate was then washed with diethyl ether and dried under vacuum.

Copolymer **4** was then reacted with AIBN (10 molar excess) in DMSO (15% w/w solution of polymer) under Ar for 3 h at 70 °C in a sealed ampule to remove dithiobenzoate (DTB) end groups. The reaction mixture was isolated by precipitation with acetone; the precipitate was washed with diethyl ether and dried under vacuum to yield copolymer **5** (Scheme 2).

Copolymers 6–8 with Dox and/or Propargyl Groups. Reactive copolymer **3** (100 mg, 58.6 μmol TT/g of the polymer) was dissolved in DMF (0.8 mL) and propargylamine (7.6 mg, 117.2 μmol) was added to the solution. After 30 min, the polymer was isolated by precipitation with acetone/diethyl ether (3:1) followed by centrifugation; the precipitate was washed with diethyl ether and dried under vacuum to form polymer **6** (Scheme 1).

Reactive copolymer **3** (100 mg, 58.6 μmol TT/g of the polymer) was dissolved in DMF (0.8 mL); Dox.HCl (10 mg, 17 μmol) and DIPEA (3.2 μL, 18.7 μmol) were then added. After 45 min, propargylamine (3.8 mg, 58.6 μmol) was added to the reaction mixture. After another 30 min, the polymer conjugate was isolated by precipitation with acetone/diethyl ether (3:1) followed by centrifugation; the precipitate was washed with diethyl ether and dried under vacuum to form polymer **7**.

Table 1. Characteristics of Copolymers 3–8

polymer	structure	M_w [a]	M_w/M_n [a]	TT mol % [b]	Dox wt % [c]
3	p(HPMA-*co*-Ma-GFLG-TT)	41000	1.6	11.1	-
4	p(HPMA-*co*-Ma-GG-TT)-DTB	31000	1.2	n.d.	-
5	p(HPMA-*co*-Ma-GG-TT)	32500	1.2	9.6	-
6	p(HPMA-*co*-Ma-GFLG-propargyl)	41000	1.7	-	-
7	p(HPMA-*co*-Ma-GFLG-propargyl-*co*-Ma-GFLG-Dox)	43000	1.6	-	8.5
8	p(HPMA-*co*-Ma-GG-propargyl-*co*-Ma-GGGFLG-Dox)	36000	1.4	-	5.1

[a]Molecular weights were determined by SEC using RI and LS detection. [b]TT determined by UV/vis spectrophotometry in DMSO (ε_{306} = 10 280 L·mol^{-1}·cm^{-1}). [c]Dox determined by UV/vis spectrophotometry in methanol (ε_{488} = 9900 L·mol^{-1}·cm^{-1}).

Reactive copolymer **5** (16.6 mg, 10.1 μmol TT/g of the polymer) and H_2N-GFLG-Dox (**2**) (3.4 mg, 3.7 μmol) were dissolved separately in a total volume of 0.5 mL of DMF and mixed together. The progress of the reaction was monitored with HPLC. After the coupling was completed, propargylamine (6.5 mg, 101 μmol) was added to the reaction mixture to end-cap the remaining reactive groups. After 30 min, the polymer was isolated by precipitation with acetone/diethyl

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Table 2. Characteristic of polymer-peptide conjugates 9-13

polymer	structure	M_w [a]	M_w/M_n [a]	peptide wt % [b]	Dox wt % [c]	biotin wt % [d]
9	p(HPMA-*co*-Ma-GG-peptide E-*co*-Ma-GG-biotin)	56000	1.7	29.0	-	1.8
10	p(HPMA-*co*-Ma-GFLG-peptide E)	46500	2.1	15.0	-	-
11	p(HPMA-*co*-Ma-GFLG-peptide-E-*co*-Ma-GFLG-Dox)	48000	1.8	13.3	8.3	-
12	p(HPMA-*co*-Ma-GG-peptide E-*co*-Ma-GGGFLG-Dox)	39000	1.5	14.9	5.1	-
13	p(HPMA-*co*-Ma-GFLG-peptide E-*co*-Ma-GFLG-Dox-*co*-Ma-GFLG-Peg_4-biotin)	49000	2.0	13.5	8.8	2.0

[a]Molecular weights were determined by SEC using RI and LS detection. [b]The peptide content (without Peg spacer) determined by amino acid analysis. [c]Dox determined by UV/vis spectrophotometry in methanol (ε_{488} = 9900 L·mol^{-1}·cm^{-1}). [d]Determined from HPLC.

ether (3:1) followed by centrifugation; the precipitate was washed with diethyl ether and dried under vacuum to form polymer **8** (Scheme 2).

For characteristics of copolymers **3**–**8**, see Table 1.

Copolymers (9–13) with Peptide E. Biotinylated polymer **9** was prepared as described previously.[2]

Polymer **7** (7.5 mg, 4.4 μmol of propargyl groups) and peptide **1** (1.3 mg, 0.4 μmol) were dissolved in DMF (150 μL). The solution was thoroughly bubbled with Ar to remove oxygen and Ru(COD) (0.15 mg, 0.4 μmol) was added to the reaction mixture. The progress of the reaction was monitored by HPLC; all peptide azide was bound to the polymer within 6 h. The polymer–peptide conjugate was separated by SEC on a Sephadex G-25 column in water and lyophilized to yield 7 mg of polymer–peptide conjugate **11**. Conjugates **10** and **12** were prepared analogously starting with polymers **6** and **8**, respectively.

Conjugate **13** with biotin was prepared starting with polymer **7** (12.3 mg, 7.2 μmol of propargyl groups) and peptide **1** (2.2 mg, 0.66 μmol) in DMF (180 μL). The solution was thoroughly bubbled with Ar to remove oxygen and Ru(COD) (0.25 mg, 0.66 μmol) was added to the reaction mixture. After 30 min, azide-Peg_4-biotin (0.3 mg, 66 μmol) and Ru(COD) (0.25 mg, 0.66 μmol) dissolved in 15 μL of oxygen-free DMF were added. The progress of the reaction was monitored by HPLC. All peptide **1** and azide-Peg_4-biotin were bound to the polymer within 6 h. The polymer–peptide conjugate was separated by SEC on a Sephadex G-25 column in water and lyophilized to yield 10.3 mg of polymer–peptide conjugate **13** (Scheme 1). For characteristics of copolymers **9**–**13**, see Table 2.

Construction of the Expression Vector for the Protein with Fusion Sequence (scFv-K). The scFv fragment derived from the monoclonal antibody B1, scFv B1, has been obtained using a procedure similar to the previously described procedure for obtaining scFv M75 fragment.[15] The scFv B1 molecule, in the format (VH)-$(Gly_4Ser)_4$-(VL), contains 118 N-terminal residues of the heavy chain linked to 108 N-terminal residues of the light chain followed by the C-myc tag sequence EQKLISEEDL. The peptide K, i.e., $(VAALKEK)_4$, was introduced at the C-terminus of the scFv fragment, as described previously.[2] The final construct thus codes for scFv B1 mAb in the format V_H-$(Gly_4Ser)_4$-V_L-myc-K peptide-His_5.

Expression and Purification of the Fusion Protein scFv-K. For expression in *Escherichia coli* BL21(DE3) cells, a modified pET-22(b) vector was used in which the scFv coding sequence is preceded by the PelB signal sequence, allowing for translocation of the product into the periplasmic space. The His_5 tag at the C-terminus of the polypeptide is used for product isolation and purification by IMAC chromatography on Ni-CAM (Sigma). Final purification was achieved by ion exchange chromatography on a MonoS column.

Preparation of the Polymer–scFv Complexes Using Coiled Coil Interactions. Polymer–peptide conjugates **9**–**13** were dissolved in PBS buffer (1 mg/mL) and mixed with the recombinant protein scFv-K (0.84 mg/mL) to obtain molar ratios of 1:1, 2:1, and 4:1 between peptide K and peptide E. Polymer–peptide conjugates and scFv-K were incubated for 20 min at room temperature to prepare the coiled coil complexes. Formation of the complexes was verified by SEC and by sedimentation analysis as described in our previous paper.[2]

Cell Lines. The following cell lines of mouse origin were used: BCL1, a spontaneous B cell leukemia of BALB/c origin, which expresses epitope IgM recognized by B1 mAb, and 38C13, a murine B cell lymphoma of C3H/HeN origin without an epitope recognized by B1 mAb. Both cell lines were cultured in RPMI 1640 medium enriched with L-glutamine (4 mM), sodium pyruvate (1 mM), 2-mercaptoethanol (0.05 mM), HEPES (10 mM), penicillin (100 Units/mL), streptomycin (100 μg/mL) and 10% (v/v) heat inactivated fetal bovine serum (FBS).

Proliferation Assay *in Vitro*. The cytostatic activity of the conjugates was assessed using the [^{3}H] thymidine incorporation assay. BCL1 (0.5×10^4/well) or 38C13 (0.25×10^4/well) cells in the standard cultivation medium were seeded into 96-well flat-bottom microtiter plates. ScFv-K/polymer complexes, polymer conjugates without targeting structure or free doxorubicin were then added to the wells (in triplicate) to achieve the desired Dox concentrations (0.0016–0.4 μg/mL). The controls were incubated in standard cultivation medium only. The plates were incubated in 5% CO_2 at 37 °C for 72 h. Next, 18.5 kBq (0.5 μCi) of [^{3}H]thymidine was added to each well for the last 6 h of incubation. The cells were then collected onto glass fiber filters using a cell harvester and the radioactivity was measured in a scintillation counter. All IC_{50} values (concentration of a drug that inhibits proliferation to 50% of controls) are means of at least three independent experiments.

Flow Cytometry Analysis. The scFv-K/polymer complexes were added to the cells (BCL1 or 38C13 as negative control). Cells were incubated with either the complexes, the scFv-K alone or biotinylated B1 mAb for 20 min on ice in the dark and afterward washed three times with FACS solution (PBS with 2% FBS and 2 mM EDTA). The cells were then incubated with 1:1000 streptavidin-APC, streptavidin-FITC or with antimyc antibody conjugated with Alexa Fluor 647 for 20 min on ice in the dark. The cells were washed three times with FACS solution and then immediately analyzed on a BD LSR II flow cytometer. The mean fluorescence intensity (MFI) of APC/FITC/Alexa Fluor 647 was determined in live (Hoechst33258-negative) cells, and GateLogic software was used for analysis of the data. The concentrations of conjugates are expressed as the concentration of scFv fragment in the sample.

Statistical Analysis. The results demonstrate an average ± standard deviation or representative result of at least three independent experiments. The unpaired Student's *t* test was used to assess if the differences observed between the various experimental groups were statistically significant ($p < 0.05$).

RESULTS AND DISCUSSION

Peptide Synthesis. The coiled coil peptide E was prepared by solid phase condensation of protected heptapeptide fragments, as described previously.[2]

Synthesis of tetrapeptide-Dox derivative **2** was based on reaction of Trt-GFLG with doxorubicin followed by removal of the trityl group as described earlier.[12] According to the original work, Trt-GFLG was prepared using a laborious multistep solution synthesis that required lengthy purifications of the intermediate products after every reaction step. Therefore, we decided to prepare the protected tetrapeptide on a solid phase support. However, use of the most common resins with acid-labile linkers does not allow for cleavage of *N*-tritylated peptide without loss of the trityl group. Instead, we synthesized Trt-GFLG on 4-Fmoc-hydrazinobenzoyl AM Novagel resin,

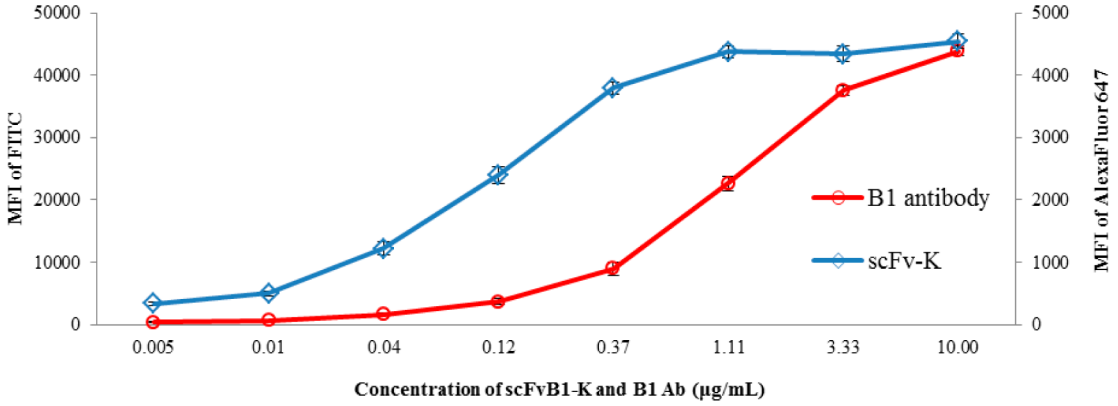

Figure 1. Binding activity of the recombinant protein scFv-K and B1 mAb to BCL1cells evaluated by flow cytometry. BCL1 cells were incubated with biotinylated B1 mAb or recombinant protein scFv-K for 20 min. BCL1 cells were then incubated with streptavidin-FITC or antimyc-Alexa Fluor 647 antibody to detect bound B1 mAb or scFv-K protein for 20 min, respectively. Cells were intensively washed with FACS solution and analyzed by the BD LSR II flow cytometer. The fluorescence intensity of FITC or Alexa Fluor 647 on live (Hoechst33258-negative) cells was analyzed.

enabling cleavage of the protected peptide by reduction with Cu(II) acetate in the presence of pyridine. Although this method was much faster and easier than the original method, the benefits have a trade-off of a relatively low yield of 28% (based on the resin substitution) that may be caused by poor solubility of the reagents and the product in the water-dioxane mixture. There are a number of publications describing the successful preparation of peptide amides, esters and thioesters using the hydrazinobenzoyl resin and corresponding amines and alcohols, respectively.[16−18] Unfortunately, we have not found any examples of the preparation of peptide acids with this resin.

Polymerizations. The majority of the HPMA copolymers used for the preparation of polymer drug carriers and their conjugates with drugs have been prepared by free radical solution polymerization. Although very encouraging biological activity data were obtained with the polymer−drug conjugates prepared this way, we are aware that broad distribution of molecular weights may have undesired consequences, namely, in terms of the pharmacokinetics of such polymer therapeutics (or diagnostics).

Recently, several papers have reported the preparation of HPMA copolymers by RAFT polymerization.[19−22] The resulting copolymers generally have a much lower polydispersity index compared to those prepared by free radical polymerization. It is obvious that the molecular weight distribution has a significant impact on the biological behavior of the copolymers. Therefore, we prepared the polymer−Dox conjugates described in this paper using both types of polymerization. In the case of polymer **4** prepared by RAFT copolymerization, the terminal DTB group was removed by reaction with excess AIBN prior to the reaction of the resulting polymer **5** with peptide **1**. Removal of the DTB groups is quite important due to the reactivity of these groups with nucleophiles; DTB might react in the subsequent step, e.g., with lysine amino groups of peptide **1**. This would lead to undesired branching of the polymer−peptide conjugate. Moreover, the presence of DTB functionality in the copolymer containing TT does not allow for spectrophotometric determination of the content of reactive TT groups due to the overlapping UV spectra of the two chromophores.

We successfully prepared copolymer **4** containing HPMA and Ma-GG-TT repeating units using RAFT polymerization. Unfortunately, our attempts to prepare an analogous copolymer of HPMA with Ma-GFLG-TT led to products with relatively high polydispersity and low yields. Therefore, we used copolymer **4** for the attachment of Dox derivative **2** containing an enzymatically degradable GFLG spacer.

Polymer Analogous Reactions. Reactive polymer precursors **3** and **5** were used as starting materials for all polymer analogous reactions. Copolymer **3** was reacted with propargyl amine to yield copolymer **6**. Copolymer **7** was obtained by reaction of polymer **3** with Dox followed by the addition of propargyl amine. Reaction of copolymer **5** with tetrapeptide-Dox derivative **2** yielded polymer−Dox conjugate **8** with a relatively narrow molecular weight distribution. Biotinylated polymer peptide conjugate **9** was prepared by reaction of the reactive copolymer p(HPMA-*co*-Ma-GG-TT) with *N*-(2-aminoethyl)biotinamide and propargyl amine followed by copper-catalyzed azide−alkyne cycloaddition ("click" reaction) of peptide **1** as described previously.[2] The "click" reaction between peptide azide **1** and the propargyl groups of polymers **6** and **7** resulted in polymer−peptide conjugates **10** and **11**, respectively. Analogously, the "click" reaction between peptide **1** and polymer **8** afforded polymer peptide conjugate **12** with low polydispersity. Because our initial attempts to use copper-catalyzed "click" chemistry for attachment of peptide **1** to doxorubicin-containing polymers **7** and **8** were unsuccessful due to the formation of copper−doxorubicin complexes,[23,24] the ruthenium-based catalyst,[25] namely, pentamethylcyclopentadienyl(cyclooctadiene) ruthenium(II) chloride, was used in the synthesis. This catalyst enabled successful attachment of the azide peptide **1** to the polymer−Dox conjugates in quantitative yields.

Binding Activity of the scFv-K Protein to BCL1 Cells. First, we checked the receptor-specific binding activity of the B1 mAb and the recombinant protein scFv-K to BCL1 leukemia cells using flow cytometry (Figure 1). Formation of the biotin/streptavidin complex was used for detection of cell surface-bound mAb or polymer conjugates. The recombinant protein scFv-K bound very well to target BCL1 cells, to a similar extent as intact B1 mAb. Unfortunately, a quantitative comparison of the binding efficacy of the B1 mAb, the scFv-K and the scFv-K/polymer complexes was very difficult due to different amounts of biotin on B1 mAb and the polymer and due to the requirement of a different method of detection of the free scFv-K protein bound to the cells (detected with antimyc-Alexa Fluor 647 antibody).

Noncovalent Polymer–scFv Complex Formation and Its Binding Activity to BCL1 Cells. The polymer–protein complexes were prepared by simple mixing of the polymer–peptide conjugates **9**–**13** with the corresponding amounts of the recombinant protein scFv-K. The formation of the complexes due to the coiled coil interactions between peptides E and K was confirmed by SEC (Figure 2) as we have already described.[2]

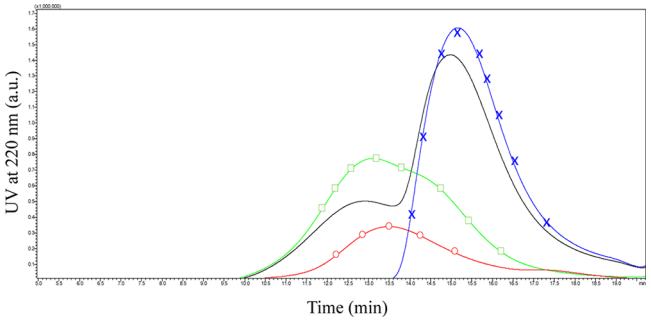

Figure 2. SEC chromatograms (UV detector, 220 nm, MicroSuperose 12, 0.05 M phosphate buffer with 0.15 M NaCl, pH 6.5, 0.1 mL/min) of the product of the mixture of polymer **9** (red circles) with the K-scFv fragment (blue crosses) in two different molar ratios of peptide E/peptide K, 1:1 (green squares) and 1:4 (black line).

The binding activity of the whole scFv-K/polymer **9** complexes to target cells was evaluated for conjugates differing in ratios of scFv-K/polymer peptide E (Figure 3). Figure 3 shows that an excess of the targeting ligand scFv-K relative to the concentration of peptide E in the mixture led to a decrease in the fluorescence associated with the cells. It is very likely that the free protein competes with the scFv-K/polymer complexes in binding to the cell receptors; this may explain the lower level of cell binding of the complexes in presence of excess of the free scFv-K.

Selectivity of Binding Activity of the Conjugates to BCL1 Cells *in Vitro*. The *in vitro* binding activity of scFv-K/polymer complexes of polymer **13** and scFv-K to BCL1 leukemia was confirmed by flow cytometry. Polymer **13** carried biotin in addition to the cytostatic drug doxorubicin. The biotin bound to the polymer was detected with streptavidin-APC. Whole complex scFv-K/polymer **13** bound to BCL1 cells very well and in a concentration-dependent manner. 38C13 B lymphoma cells, the cell line without the epitope recognized by B1 mAb, were used as a control to confirm high specificity of the scFv-K binding. Binding to these cells was not observed for either scFv-K or for the whole targeted complex scFv-K/polymer **13** (Figure 4). The binding activity of scFv-K/polymer **13** complexes was measured directly using the fluorescence of doxorubicin conjugated to polymer carrier (data not shown). Although Dox is a relatively poor fluorophore, we have observed the same dependence of fluorescence intensity on increasing concentration of scFv fragment of B1 antibody using BCL1 cells. Almost no increase in the fluorescence intensity with increasing ligand concentration was observed in the case of the control 38C13 cells.

Table 3. Cytostatic Activity of the scFv-Targeted and Nontargeted Polymer–Dox Conjugates 11 and 12, PK1 and Free Dox

substance	IC_{50} (μg/L)[a]	
	BCL1	38C13
Polymer 11	146 ± 42	826 ± 35
Polymer 12	197 ± 23	621 ± 49
Polymer 11 + scFv-K	2 ± 1	1065 ± 135
Polymer 12 + scFv-K	3 ± 1	899 ± 18
Dox	1.2 ± 0.3	8 ± 5
PK1	1279 ± 273	4074 ± 2296

[a]IC_{50} values in μg/L, concentration of Dox in copolymer.

The results presented in Figure 4 show that the binding activity of the scFv-targeted conjugate to BCL1 cells is concentration dependent. The binding affinity is 50 − 100 times higher than that of the control 38C13 cells (at the same scFv concentration), thus demonstrating high specificity of the targeted polymer for BCL1 cells.

Cytostatic Activity of the scFv-Targeted Polymer–Dox Complexes. Dox-containing polymer–peptide conjugates **11** and **12**, as well as the corresponding scFv-K/polymer complexes, were tested in proliferation assay using the receptor-positive murine B cell leukemia line BCL1 and murine B cell lymphoma 38C13 as a negative control. As a nontargeted control, the polymer–Dox conjugate called PK1 (prepared as described previously)[13] and consisting of HPMA and Ma-GFLG-Dox (2 mol %) repeating units was used. This conjugate successfully passed phases I and II of clinical trials.[26] We considered the polymer PK1 to be the "gold standard" of nontargeted polymer cytostatic drugs. The results of the cytostatic activities are summarized in Table 3 and Figure 5.

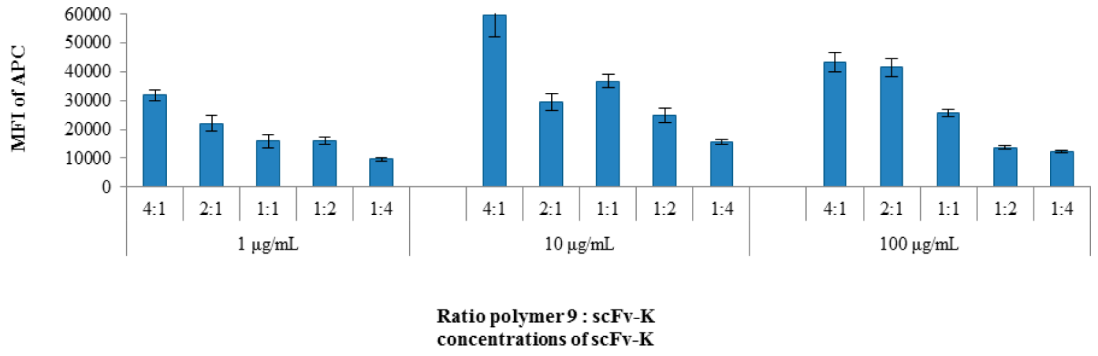

Figure 3. Binding of the scFv-K/polymer **9** bearing biotin and targeted to BCL1 cells at various molar ratios of the peptide K/peptide E was evaluated by flow cytometry. BCL1 cells were incubated with scFv-K/polymer **9** complexes for 20 min and afterward cells were incubated with streptavidin-APC for the next 20 min. The cells were intensively washed with FACS solution and analyzed by the BD LSR II flow cytometer. The fluorescence intensity of APC bound to scFv-K/polymer **9** complexes in live (Hoechst33258-negative) BCL1 cells was analyzed.

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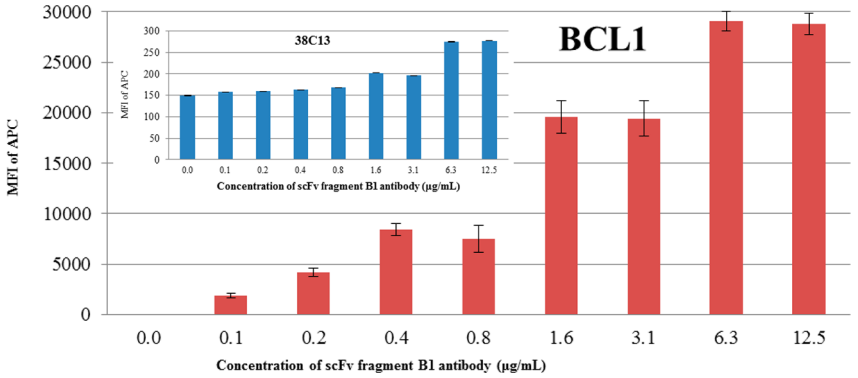

Figure 4. Binding activity of the scFv-K/polymer **13** complexes to BCL1 and 38C13 (inset) cells was evaluated by flow cytometry based on fluorescence of APC (662 nm). BCL1 cells were incubated with scFv-K/polymer **13** complexes for 20 min. The molar ratio of the peptides K: E was 1:2. Next, the cells were incubated with streptavidin-APC for 20 min to detect biotin bound on polymer **13**. The cells were extensively washed and analyzed on a BD LSR II flow cytometer. The fluorescence intensity of APC bound to scFv-K/polymer **13** complexes in live (Hoechst33258-negative) BCL1 and 38C13 cells was analyzed.

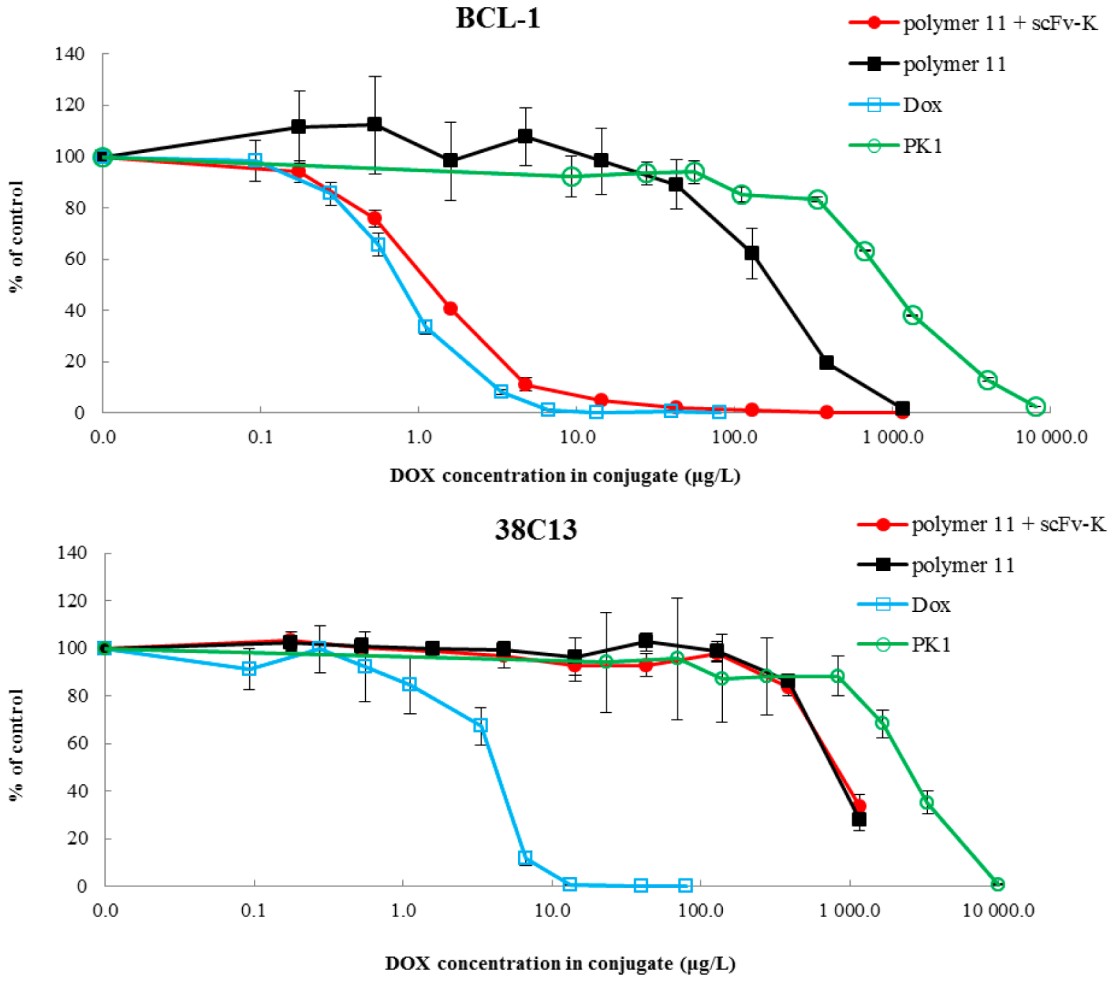

Figure 5. Proliferation of BCL1 and 38C13 cells in the presence of scFv-targeted and nontargeted polymer **11** and free Dox. The cells were incubated with scFv-K/polymer complexes, polymer without targeting structure, PK1 or free doxorubicin for 72 h. Proliferation of the cells was determined using the [^{3}H] thymidine, which was added to the cells for last 6 h of incubation. The radioactivity of samples after harvesting the cells was determined using a scintillation counter.

Polymers **11** and **12** without the targeting ligand scFv-K exhibited low cytostatic activity that was comparable to that of the control polymer-Dox conjugate PK1. A significant increase of approximately 2 orders of magnitude in cytostatic activity of the polymer−Dox conjugates **11** and **12** against BCL1 cells was observed upon addition of the targeting scFv-K ligand. The IC_{50} values of polymers **11** and **12** decreased from 146 to 2 μg/L and from 197 to 3 μg/L, respectively (see Table 3 and Figure 5), which is similar to the value obtained for free Dox. The lowest cytotoxic effect was found for the PK1 control polymer.

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No significant difference in cytotoxicity of the targeted and nontargeted polymer drugs was observed in the case of treatment of the 38C13 cells used as a negative control, which lack the appropriate antigen recognized by scFv-K. Here, a slight decrease in the cytotoxic activity (increase in IC_{50}) of the scFv-modified polymer conjugate can be ascribed to slower uptake of the more bulky conjugate by 38C13 cells that do not express the cognate antigen. The results clearly demonstrate that antigen-specific delivery of the polymer-drug conjugate to the target cells significantly increases the cytostatic activity of these conjugates *in vitro*.

As expected, a very small difference in cytostatic activity of the polymers **11** (prepared by classical radical polymerization) and **12** (prepared by RAFT) was found *in vitro*. In this case, all molecules of the conjugates are in contact with cancer cells and can exhibit cytostatic effects. Conversely, under *in vivo* conditions, polymer **12** with narrower molecular weight distribution might exhibit better biological activity than the "classical," more polydisperse polymer **11**. Lower-molecular-weight fractions of polymer **11** (necessarily present in the conjugate with higher polydispersity) can be rapidly excreted by glomerular filtration,[27] thus decreasing the effective concentration of the polymer drug in the organism. In addition, we can expect that the more uniform polymer **12** prepared by the RAFT technique will exhibit better and more specific pharmacokinetics, allowing for a more precise study of the mechanism of action of the conjugate. We believe that the forthcoming *in vivo* experiments will confirm our expectations.

CONCLUSION

The water-soluble polymer conjugates bearing cancerostatic Dox and peptide E were synthesized using click chemistry catalyzed by pentamethylcyclopentadienyl(cyclooctadiene) ruthenium(II) chloride. Peptide E can form a coiled coil heterodimer with peptide K, which is a part of the recombinant protein consisting of the scFv fragment of B1 mAb. Both B1 mAb and scFv-K bind to murine BCL1 leukemia cells with high affinity. The specific binding of the scFv-K/polymer complexes to BCL1 cells was confirmed by flow cytometry. The cytostatic activity of the targeted scFv-K/polymer-Dox complexes against BCL1 leukemia cells was approximately 100-times higher than that of the nontargeted polymer conjugate alone. There was no difference in the cytostatic activity of the targeted scFv-K/polymer complexes and the nontargeted polymers in 38C13 B-cell lymphoma that do not express the antigen recognized by B1 mAb.

The results of this study demonstrate that HPMA copolymer-based conjugates targeted with recombinant scFv fragment attached specifically to the polymer-drug carrier via coiled coil heterodimers can be used as targeted polymer anticancer drugs.

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Notes

The authors declare no competing financial interest.

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