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POLYMER CANCEROSTATICS TARGETED BY RECOMBINANT ANTIBODY FRAGMENTS TO GD2-POSITIVE TUMOR CELLS

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KEYWORDS

polymer cancerostatics, drug targeting, scFv, bungarotoxin, doxorubicin, GD2 antigen

ABSTRACT

A water-soluble polymer cancerostatic actively targeted against cancer cells expressing a disialoganglioside antigen GD2 was designed, synthesized and characterized. A polymer conjugate of an anti-tumor drug doxorubicin with a *N*-(2-hydroxypropyl)methacrylamide-based copolymer was specifically targeted against GD2 antigen-positive tumor cells using a

recombinant single chain fragment (scFv) of an anti-GD2 monoclonal antibody. The targeting protein ligand was attached to the polymer-drug conjugate either via a covalent bond between the amino groups of the protein using a traditional non-specific aminolytic reaction with a reactive polymer precursor or via a non-covalent but highly specific interaction between bungarotoxin covalently linked to the polymer and the recombinant scFv modified with a C-terminal bungarotoxin-binding peptide. The GD2 antigen binding activity and GD2-specific cytotoxicity of the targeted non-covalent polymer-scFv complex proved to be superior to the covalent polymer-scFv conjugate.

1. INTRODUCTION

Conjugates of hydrophilic polymers with cytostatic drugs offer numerous advantages in the treatment of malignancies compared to low-molecular weight cancerostatics.[1] The polymer conjugates generally exhibit higher solubility, prolonged blood circulation and increased accumulation in solid tumors. The choice of a specific targeting ligand can further increase the therapeutic potential of these polymer-drug conjugates. Among the numerous targeting ligands described, antibodies and antibody fragments are the most efficient. However, well-defined covalent attachment of a complex protein molecule to a multivalent polymer carrier is challenging. Therefore, we have investigated various covalent and non-covalent methods of polymer-protein conjugation. Recently, we developed[2,3] an anchoring system based on the formation of a coiled-coil heterodimer between two complementary peptide sequences. The peptide $(IAALESE)_2$-IAALESKIAALESE was covalently attached to a synthetic hydrophilic copolymer based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) bearing a cytostatic drug doxorubicin (Dox) and/or a diagnostic label; peptide IAALKSKIAALKSE-$(IAALKSK)_2$ was incorporated into the C-terminal part of a recombinant single chain antibody fragment (scFv) of

the B1 monoclonal antibody that specifically binds to murine BCL1 leukemia cells. The polymer-protein complex formed upon mixing of the polymer-Dox conjugate with scFv exhibited significantly higher specific cytotoxicity against murine BCL1 leukemia in vitro and markedly increased anti-leukemia efficacy in vivo compared with the non-targeted polymer-Dox conjugate (without the scFv ligand).

Tumor associated carbohydrate antigens represent attractive targets for therapy.[4] One such antigen is disialoganglioside GD2, which poses restricted expression in normal tissue and is primarily expressed on the cell surface of various tumor tissues including neuroblastoma, most melanoma lesions, sarcoma, glioma, retinoblastoma, small cell lung cancer, brain tumors, osteosarcoma, rhabdomyosarcoma, Ewing's sarcoma in children and adolescents, as well as liposarcoma, fibrosarcoma, leiomyosarcoma and other soft tissue sarcomas in adults.[5–7] Moreover, its presence in breast cancer stem cells has recently been reported.[8,9] The total incidence of GD2-positive tumors in the USA is >200 000 annually. These cancers have a high mortality rate (20–80%), where 60–100% are possible candidates for anti-GD2 targeted therapy.[10]

In view of the GD2 therapeutic potential, this work focused on actively targeted polymer anticancer drugs based on HPMA copolymers as a polymer carrier (P), Dox as an anticancer drug and the recombinant scFv fragment of anti-GD2 antibody as a targeting moiety. We utilize a non-covalent interaction between α-bungarotoxin (BTX) covalently attached to the HPMA-based copolymer with Dox and bungarotoxin-binding peptide WRYYESSLEPYPD (Bp) linked to the C-terminus of the recombinant scFv of anti-GD2 antibody. The antigen-specific cell binding and cytotoxicity of this drug delivery system was investigated and compared with corresponding covalent polymer-drug-protein conjugates (P-Dox-scFv) prepared via the

traditional aminolytic reaction between a reactive polymer precursor and the ε-Lys amino groups of the protein.

It was hypothesized that the non-covalent conjugate P-Dox-BTX / scFv-Bp might exhibit superior binding activity to GD2 antigen and increased cytotoxicity against malignant GD2-positive cell lines compared with the covalent conjugate P-Dox-scFv and with the non-targeted P-Dox conjugate. The approach utilizing formation of the non-covalent complex between the recombinant scFv-Bp and P-Dox-BTX leads to a structurally better-defined product compared with the covalent conjugate P-Dox-scFv, which may substantially facilitate approval of targeted nanotherapeutics for eventual clinical applications by regulatory authorities.

2. EXPERIMENTAL SECTION

2.1 Materials

Methacryloyl chloride, 1-aminopropan-2-ol, 3-aminopropanoic acid, 4,5-dihydrothiazole-2-thiol, dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N,N'*-dicyclohexylcarbodiimide (DCC), 2,2'-azobis(isobutyronitrile) (AIBN), 4-cyano-4-thiobenzoylsulfanylpentanoic acid, *N*-(2-aminoethyl)biotinamide hydrobromide (biotin-NH_2), *N,N*-dimethylacetamide (DMA), dimethyl sulfoxide (DMSO) and tert-butyl alcohol were purchased from Sigma-Aldrich. 2,2'-Azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70) was purchased from Wako Chemicals Europe GmbH, Germany. 2-Chlorotrityl chloride resin, protected amino acid derivatives and were purchased from Iris Biotech, GmbH, Germany. 3-Amino-1-(11,12-didehydrodibenzo[b,f]azocin-5(6H)-yl)propan-1-one (Dbco-NH_2) was purchased from Click Chemistry Tools (AZ, USA). Doxorubicin hydrochloride was purchased from Meiji Seika, Japan and BTX was from Alomone Labs, Israel.

All other reagents and solvents were purchased from Sigma-Aldrich, Prague, Czech Republic. All chemicals and solvents were analytical grade.

2.2 HPLC Monitoring of Polymer-Analogous Reactions

Monitoring of the conjugation reactions of Dbco-NH_2, biotin-NH_2 and *N*-(5-azidopentanoyl-glycylphenylalanylleucylglycyl)doxorubicin (Az-GFLG-Dox) to the reactive polymer precursors was performed by HPLC using a 100 × 4.6 mm Chromolith Performance RP-18e column (Merck, Germany) and 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).

2.3 Mass spectrometry and Size-Exclusion Chromatography (SEC)

The molecular mass of the monomers and peptide derivatives was determined using mass spectrometry performed on an LCQ Fleet mass analyzer with electrospray ionization (ESI MS) (Thermo Fisher Scientific, Inc., MA, USA). The determination of the molecular weights and polydispersity of the copolymers was performed by SEC on a HPLC system (Shimadzu, Japan) equipped with UV, differential refractive index, and multi-angle light scattering (LS) DAWN Heleos II (Wyatt Technology Corp., USA) detectors using a TSK 3000 SW_{XL} column (Tosoh Bioscience, Japan) (80% methanol, 20% 0.3 M acetate buffer pH 6.5) at a flow rate of 0.5 mL/min. The association behavior of the BTX and polymer-BTX conjugates with scFv-Bp and conjugation of the proteins to copolymers was studied using a MicroSuperose® 12 column (30 cm × 3.2 mm, 0.01 M phosphate buffer with 0.15 M NaCl, pH 7.4) at a flow rate of 0.1 mL/min.

2.4 UV/VIS Spectrophotometry

The contents of thiazolidine-2-thione (TT) groups, doxorubicin, dithiobenzoate (DTB) end groups and aza-dibenzocyclooctyne (Dbco) groups were determined spectrophotometrically on a Helios Alpha UV/VIS spectrophotometer (Thermospectronic, UK) using the following absorption coefficients ε_{TT} = 10 300 L mol^{-1} cm^{-1} (305 nm, methanol), ε_{Dox} = 8200 L mol^{-1} cm^{-1} (488 nm, water), ε_{DTB} = 12 100 L mol^{-1} cm^{-1} (302 nm, methanol), ε_{Dbco} = 13 000 L mol^{-1} cm^{-1} (292 nm, methanol).

2.5 Synthesis of the Monomers

2.5.1 *N*-(2-hydroxypropyl)methacrylamide (HPMA)

N-(2-Hydroxypropyl)methacrylamide (HPMA) was prepared by the reaction of methacryloyl chloride with 1-aminopropan-2-ol in dichloromethane.[11] Methacrylamidopropanoic acid (Ma-AP-OH) was prepared by the reaction of methacryloyl chloride with 3-aminopropanoic acid in an aqueous alkaline medium.[12] 3-Methacrylamidopropanoylthiazolidine-2-thione (Ma-AP-TT) was synthesized by the reaction of Ma-AP-OH with 4,5-dihydrothiazole-2-thiol in the presence of dimethylaminopyridine. The synthesis was performed according to a previously described method[13] using EDC instead of DCC allowing removal of the water-soluble urea derivative by extraction of the organic solution with water. The monomer was characterized using HPLC (single peak) and ESI MS (calculated 258.3, found 259.1 M+H).

2.5.2 5-Azidopentanoylglycylphenylalanylleucylglycine (Az-GFLG-OH)

Az-GFLG-OH was prepared via manual solid phase peptide synthesis on 2-chlorotrityl chloride resin (0.5 g, loading 1 mmol/g) using 0.2 M solutions of AA (5-azidopentanoic acid, Fmoc-Gly-OH, Fmoc-Phe-OH, Fmoc-Leu-OH). The product was cleaved from the resin with a

30% solution of HFIP in DCM yielding 121 mg (0.23 mmol, 46%) of the peptide azide. ESI MS (calculated 531.6, found 554.6 M+Na).

2.5.3 N-(5-azidopentanoylglycylphenylalanylleucylglycyl)doxorubicin (Az-GFLG-Dox)

Az-GFLG-OH (50 mg, 0.094 mmol), 1-hydroxysuccinimide (12 mg, 0.104 mmol) and DCC (23 mg, 0.113 mmol) was reacted in THF (2 mL) at 4°C overnight. A drop of acetic acid was added to the mixture, then the precipitated *N,N'*-dicyclohexylurea was removed by filtration 30 min later and the filtrate was evaporated to dryness. The solid was dissolved in DMA (0.5 mL) and added to a solution of doxorubicin hydrochloride in DMA (0.5 mL), followed by DIPEA (0.094 mmol, 16 μL). The progress of the reaction was followed by TLC (silica gel, chloroform/methanol, 9:1). After 1 h, the reaction mixture was chromatographed on a column (silica gel, chloroform/methanol, 9:1). The collected fractions with the required product (according to HPLC) were evaporated to dryness; the residue was dissolved in methanol and precipitated to diethyl ether to yield 80 mg (0.077 mmol, 82%) of the title compound. ESI MS (calculated 1042.4, found 1065.7 M+Na).

2.5.4 Reactive Polymer Precursor P-TT

Copolymer poly(HPMA-*co*-Ma-AP-TT) (P-TT) was prepared by reversible addition-fragmentation chain transfer (RAFT) copolymerization of HPMA (1.5 g, 10.5 mmol, 88 mol%) and Ma-AP-TT (369 mg, 1.43 mmol, 12 mol%) using V70 (0.03 mmol, 9.17 mg) as an azo initiator and (1-cyano-1-methyl-ethyl) benzenecarbodithioate (0.06 mmol, 13.2 mg) as a chain transfer agent (CTA). The molar ratio of monomers/CTA/initiator used was 400:2:1. The polymerization mixture was dissolved in tert-butyl alcohol with 15% of DMSO (17 mL, 0.9 M solution of monomers), transferred into a glass ampule, bubbled with Ar and sealed. After 16 h at 40°C, the product was isolated by precipitation with acetone, then washed with diethyl ether and

dried under vacuum. The copolymer was 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) ω-end groups.[14] The reaction mixture was isolated by precipitation with acetone, the precipitate was washed with diethyl ether and dried under vacuum to yield copolymer *P-TT* (1.1 g, 59%). Molecular parameters of the product were M_w = 27200, M_w/M_n = 1.03. Content of the reactive TT groups was 11.6 mol%.

2.5.5 P-TT-biotin-Dbco

Copolymer P-TT (100 mg, 75 μmol TT) in DMA (0.8 mL) and *N*-(2-aminoethyl)biotinamide hydrobromide (2.3 mg, 6.3 μmol) in DMA (0.2 mL) were mixed together and reacted for 3 h at 25°C. Then Dbco-NH_2 (8.8 mg, 32 μmol) was dissolved in the reaction mixture and DIPEA (5 μL, 32 μmol) was added. The reaction was completed in 40 min (as indicated by HPLC) and the polymer product was twice precipitated into acetone/diethyl ether (1:1) to yield 102 mg of the title copolymer P-TT-biotin-Dbco. The biotin content was 1.0 mol% as determined by HABA assay.[15]

2.5.6 P-Dox

A solution of Az-GFLG-Dox (4.2 mg, 4 μmol) in DMA (50 μL) was added to P-TT-biotin-Dbco (18 mg, 5.7 μmol Dbco) dissolved in DMA (150 μL). After 2 h at 25 °C, the crude polymer was precipitated into acetone/diethyl ether (1:1), re-dissolved in PBS (2 mL) containing 1-aminopropan-2-ol (1 μL), purified on Sephadex G-25 in water and lyophilized to yield 16.4 mg of the title copolymer. The polymer conjugate contained 10.6% w/w of Dox.

2.5.7 P-Dbco-BTX

BTX (1 mg, 0.125 μmol) was dissolved in 40 μL of 0.01 M phosphate buffer with 0.15 M NaCl pH 7.4 (PBS) and mixed with equal volume of PBS solution of copolymer P-TT-biotin-

Dbco (2 mg, 0.64 μmol TT). The aminolytic reaction was terminated after 16 h by addition of 1-aminopropan-2-ol (0.08 μL in 16 μL of PBS). The title polymer conjugate was purified via Sephadex G-25 in PBS and concentrated to final volume of 150 μL using an Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-10 membrane (cut off 10 kDa).

2.5.8 P-BTX-Dox

Az-GFLG-Dox (0.4 mg, 0.38 μmol) in DMSO (6 μL) was added to the solution of polymer conjugate P-Dbco-BTX (3 mg in 150 μL of PBS) under stirring. A small fraction of undissolved Dox derivative was removed by centrifugation 16 h later. The polymer conjugate contained 7.7% w/w of Dox.

2.5.9 P-Dbco-scFv

A solution of scFv (preparation is described below) in PBS (9 mg in 2.5 mL) was mixed with a PBS solution of polymer P-TT-biotin-Dbco (18 mg, 0.5 mL). The reaction mixture was kept for 16 h at 25°C and the solution was used for subsequent reactions.

2.5.10 P-scFv-Dox

Az-GFLG-Dox (3.5 mg, 3.35 μmol) in DMSO (50 μL) was added to the solution of polymer conjugate P-Dbco-scFv (27 mg in 3 mL of PBS) under stirring. A small fraction of undissolved Dox derivative was removed by centrifugation 16 h later. The polymer conjugate contained 9.1% w/w of Dox.

2.5.11 Non-Covalent Complex P-BTX-Dox / scFv-Bp

The polymer conjugate P-BTX-Dox (1.8 mg in 0.3 mL PBS) and equimolar amount (molar ratio BTX/Bp = 1/1) of recombinant protein scFv-Bp (1.2 mg in 0.3 mL) were mixed at 25°C to yield the non-covalent polymer-protein complex within 10 min. The resulting solution was used for flow cytometry and cytotoxicity studies.

2.5.12 Non-covalent complex P-Dbco-BTX / scFv-Bp

The complex was prepared by the same procedure as described above.

2.6 Cloning, Expression and Purification of scFv Molecules

DNA encoding variable domains of heavy and light chains of anti-GD2 mAb hu3F8 were chemically synthesized according to a published sequence[16] and assembled into the scFv fragment as described previously.[3,17] The scFv anti-GD2 molecule, in the format (VH)-$(Gly_4Ser)_4$-(VL), contains 118 N-terminal residues of the heavy chain linked to 105 N-terminal residues of the light chain, followed by the C-myc tag sequence EQKLISEEDL and His_5 tag. A DNA fragment coding for 13 amino acid residues long BTX binding peptide WRYYESSLEPYPD (Bp), which displays strong non-covalent interaction with α-bungarotoxin (BTX), was introduced between the myc and his tags in the scFv (scFv-Bp). As a control we used recombinant scFv of B1 mAb, which recognizes the idiotype of surface IgM expressed on mouse BCL1 leukemia cells. The appropriate DNA fragment coding the scFv B1 was used to replace anti-GD2 sequence in the above anti-GD2 scFv molecule. This mock scFv-Bp-B1 was expressed in a bacterial system as described earlier.[3]

For a covalent mode of attachment to the polymer, the anti-GD2 scFv fragment was simply bound to polymer by aminolytic reaction.[2]

For expression, the anti-GD2 scFv-Bp coding sequence, fused to the antibody heavy chain leader sequence to allow secretion of the product into the medium, was cloned into the vector pcDNA3.1. Transfection and cultivation using the ExpiCHO system (Thermofisher) was performed according to the high titre manufacturer's manual, with minor modifications.

Purification of the recombinant product was performed on a HisTRAP 5 mL column (GE Healthcare) with the following buffers: buffer A (50 mM NaH_2PO_4 .$2H_2O$; 300 mM NaCl; pH 8.4) and buffer B (50 mM NaH_2PO_4 .$2H_2O$; 300 mM NaCl; 500 mM imidazole; pH 8.4). Conditioned media with scFv-Bp was dialyzed 3 times against 3 L of buffer A. The column was equilibrated by 10 column volumes of buffer A and washed with 10 column volumes of buffer A and 10 column volumes of buffer A + 1% buffer B. For the elution, buffer A + 10% buffer B, buffer A + 50% buffer B and 100% buffer B were used. All steps were performed at the rate of 1 ml/min at 4°C (except loading speed 0.5 ml/min). After elution, the fractions were analyzed by SDS-PAGE and western blot. The concentration of eluted protein was determined by A_{280} measurement using an extinction coefficient obtained from ExPASy, tool ProtParam.

2.7 Isothermal Titration Calorimetry (ITC)

ITC measurements were performed on a MicroCal-200 isothermal titration calorimeter. The experiment was conducted with consecutive injections of BTX or polymer-BTX conjugate solution into the calorimeter cell. The cell contained 280 µL of the binding peptide or scFv-Bp protein solution. The BTX solution was added with a 40-µL injection syringe with a tip modified to act as a stirrer. The chosen stirring speed was 750 rpm. The injection volume was set for the first injection to 0.5 µL, and 2 µL for the rest of the 19 injections. The time between injections was varied from 300 to 600 seconds to reach a baseline. The measurements were conducted at 37°C. The data were analyzed using the Microcal ORIGIN software.

2.8 Dynamic Light Scattering (DLS)

DLS was performed to characterize the individual solutions of polymer conjugate and protein, and their mixture after ITC. For this purpose, the hydrodynamic diameter of the particles, D_h, and the scattering intensity were measured at a scattering angle of θ = 173° with a Zetasizer Nano-ZS instrument, model ZEN3600 (Malvern Instruments, UK). The DTS (Nano) program was used to evaluate the data. The volume weighted value of the apparent D_h was chosen to characterize the dispersity of the solutions. Solutions were filtered prior to DLS experiments using 0.22 μm PVDF filter (Millipore).

2.9 Cells and Media

UKF-NB-3 and EL-4 cells were obtained from ATCC. The NIH-3T3 cell line was a kind gift from Dr Jan Závada (IMG). The cell line UKF-NB-3 was cultured in IMDM media (Sigma-Aldrich), while EL-4 and NIH-3T3 in DMEM media (Sigma-Aldrich), supplemented with 10% of heat inactivated FCS (Gibco, South America origin) and antibiotics Anti-Anti (Gibco). These cell lines were incubated under standard conditions of 5% CO_2 at 37°C. Maternal ExpiCHO cell culture was maintained in ExpiCHO expression medium at 37°C, 8% CO_2, 150 rpm. All cell lines tested negatively for the presence of mycoplasma.

2.10 Flow Cytometry

Cells (4×10^5) in 20 μL PBS with 1% BSA (Sigma-Aldrich) were incubated with targeted conjugate, non-targeted conjugates and controls at similar concentrations of targeting scFv either polymer-bound or in control. Non-targeted conjugates were diluted to the same concentration which was used in the targeted conjugates. Solutions of the polymer conjugates contained 16–130 μg mL^{-1} of Dox equivalent. The commercially obtained secondary antibody anti-His/FITC

(Exbio) for detection of the recombinant scFv (containing His-tag) was diluted 100 times, while streptavidin/FITC (Sigma-Aldrich) for detection of the biotinylated polymer was diluted 200 times. Incubation of samples with the cells in each step was for 30 min at 4°C. Between incubations of the cells with conjugates and staining molecules, the samples were washed twice by ice cold 1% BSA in PBS. Stained samples were analyzed by flow-cytometer (LSRFortessa, BD), with the gating strategy FSC-A, SSC-A; FSC-A, FSC-H; 561-610/20, SSC-A; 488-530/30, count. Obtained data were evaluated with FlowJo software, version 10.0 (Tree Star, Inc.).

2.11 In Vitro Cell Cytotoxicity Assay

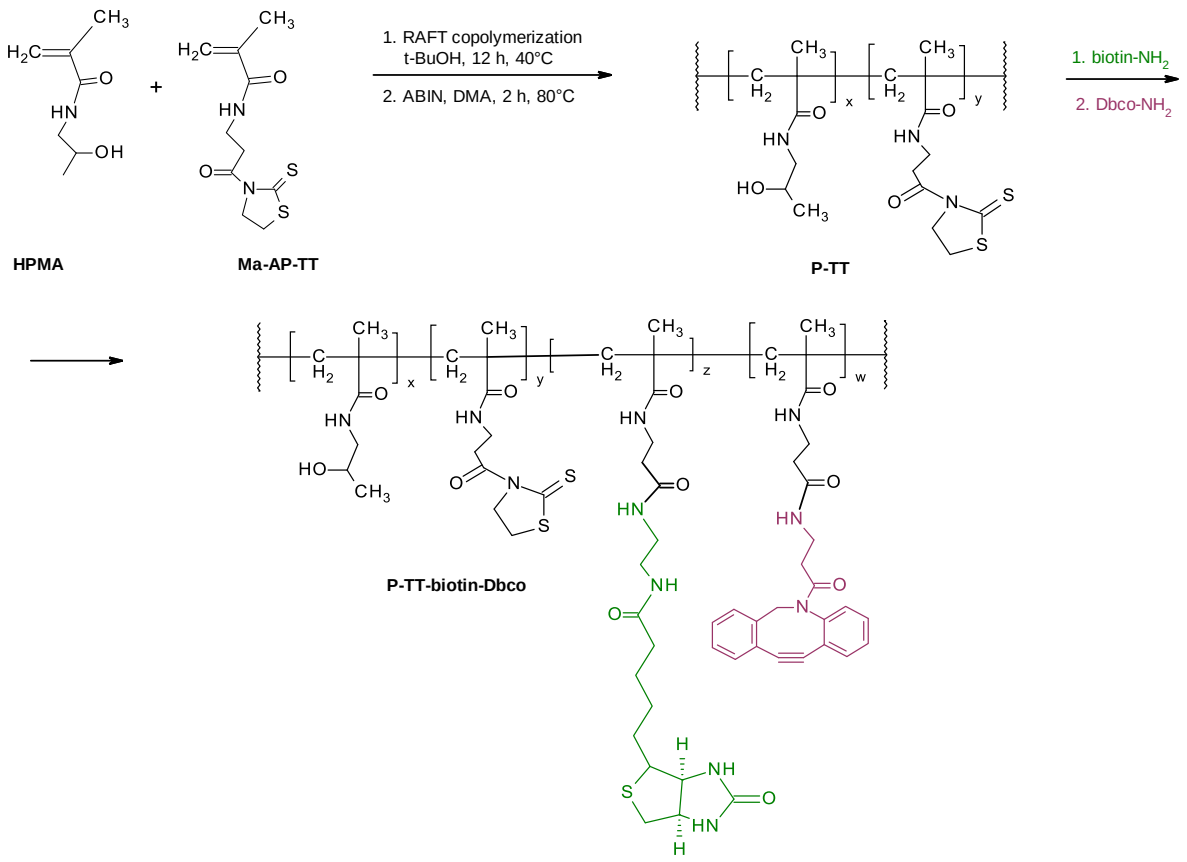
EL-4 cells were collected from an exponential growth phase culture. Cells were centrifuged for 5 min at 300 g, washed, counted in Trypan blue assay and re-suspended in fresh culture medium. The cell density was adjusted to 2×10^5 cells/ml, and 0.05 mL of this cell suspension was loaded by a repeating pipette into each well of Nunc 96-well round-bottom plates. The tested samples dissolved in growth medium were then added to the wells (50 μL/well) to achieve the required concentrations. UKF-NB-3 and NIH-3T3 cell lines were assayed similarly, with the exception that the loading cell density was 1×10^5 cells/mL and the cells were pipetted into wells 20 hours prior to adding the assayed samples. Just prior to adding the tested samples, the conditioned medium was aspirated from wells and samples dissolved in fresh growth medium in the desired concentrations were added (100 μL/well). The final well volume was 0.1 mL and cell density was 1×10^4 cells/well. Each condition was measured in triplicate. The final concentration of Dox equivalent in tested samples for EL-4 cells were in the range 0.01–144 μg/mL. The plates were cultured in 5% CO_2 for 24 and 72 h at 37°C, then 100 μL of XTT cell assay solution (Sigma-Aldrich) was added to each well, and the cell viability was measured by spectrometer at several

time points (0, 1, 2, 4 and 8 hours). The optimal time point was chosen based on the best difference between the strongest and the weakest signal of absorbance. Shown numbers of absorbance were obtained as difference data measured at two different wavelengths (490 and 655 nm) on the Infinite M200 (Tecan) and collected by software Magellan 6.0 (Tecan). Evaluation and IC_{50} determination was performed using Excel (Microsoft).

3. RESULTS AND DISCUSSION

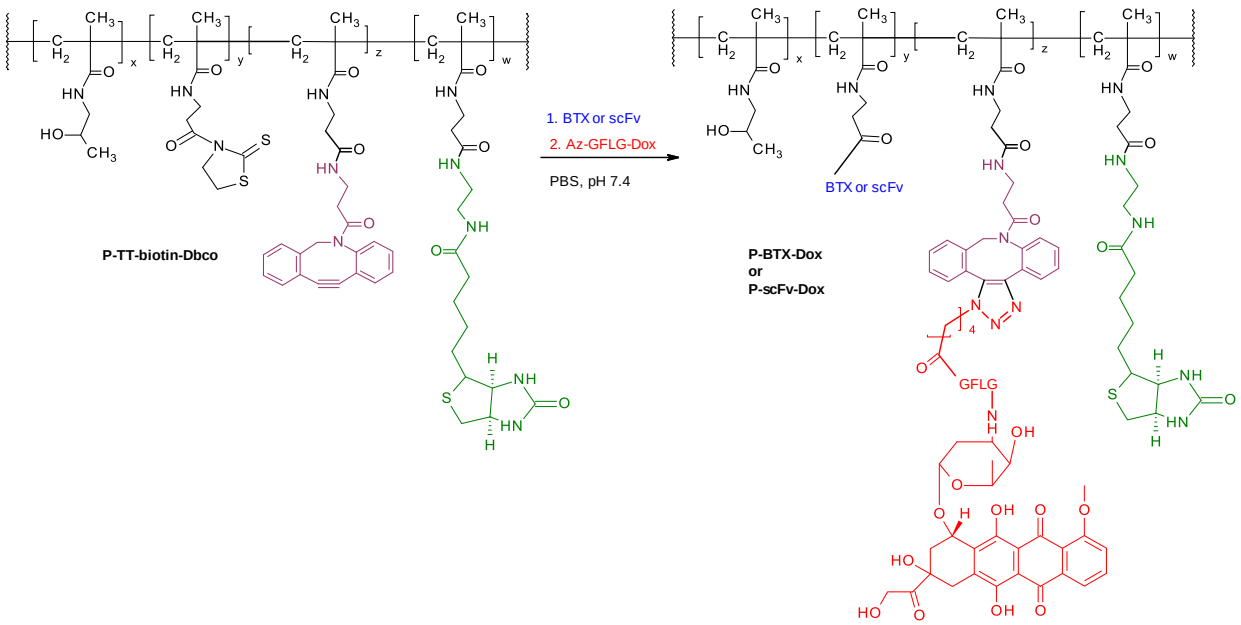
3.1 Synthesis of the Polymer Conjugates

The multivalent polymer precursor P-TT with approximately 12 mol% of reactive TT groups used for preparation of the targeted polymer conjugates was synthesized via a RAFT copolymerization of HPMA and Ma-AP-TT providing a copolymer with very low dispersity ($M_w/M_n = 1.03$). One portion of the TT groups (about 1 mol%) was modified with biotin-NH_2 to enable monitoring of the polymer using flow cytometry. Another portion of the TT groups (5 mol%) reacted with Dbco-NH_2 to yield polymer P-TT-biotin-Dbco (**Scheme 1**).



Scheme 1

The remaining TT groups (5–6 mol%) of the polymer were used for reaction with primary amino groups of α-bungarotoxin or scFv yielding the corresponding polymer-protein conjugates. In the last step, the azide derivative of Dox, Az-GFLG-Dox, containing an enzymatically cleavable tetrapeptide spacer was bound to the polymer-protein conjugates via a metal-free strain-promoted azide-alkyne cycloaddition (**Scheme 2**).



Scheme 2

The initial attempts to bind Az-GFLG-Dox to P-TT-biotin-Dbco and then conjugate the protein in PBS led to precipitation of the hydrophobic polymer in the aqueous solution providing practically zero yield of the polymer-protein conjugate. Addition of a large amount of organic solvent, such as DMSO or DMA, which was necessary for the dissolution of the precipitate resulted in total loss of biological activity (including antigen binding) of the protein.

Generally, polymers prepared via RAFT polymerization with low dispersity exhibit more favorable pharmacokinetic behavior,[18] consequently, the corresponding nanotherapeutics also have a higher therapeutic efficacy.[19] Furthermore, such polymers are more likely to have better clinical potential than more polydisperse polymers. Consequently, the polymer precursors prepared via RAFT polymerization were selected for the present study.

3.2 Recombinant Proteins and scFv-Targeted Polymer Conjugates

Non-covalent attachment of targeting protein molecules to polymer conjugates has many advantages.[20] In our previous studies,[3] we used coiled-coil interaction for non-covalent

attachment of scFv moieties to polymers. In the present study, an alternative approach was used, namely a much stronger BTX – Bp interaction.[21] This BTX-Bp pair was used for the first time in an arrangement where a recombinant PSMA-specific scFv fragment C-terminally fused to BTX was used for targeting the polymer-Bp modified adenovirus to prostate-specific membrane antigen.[22] When we attempted to produce an analogous recombinant antiGD2 scFv-BTX fusion protein, the expression yields were very low, irrespective of the expression system (bacterial or mammalian cells), so antiGD2 scFv-Bp and polymer modified with BTX were prepared as described above in the methods. As shown below, the polymer conjugate assembly realized through the non-covalent interaction BTX-Bp proved very efficient in specific targeting GD2-positive cells and was superior to the covalent conjugate.

The quality of every new batch of scFv was checked by SDS electrophoresis where no evidence of impurities was observed.

3.3 Association Behavior of BTX and Polymer-BTX Conjugates with Bp and scFv-Bp

Previously, we demonstrated that unmodified BTX and a recombinant protein consisting of scFv and BTX is able to bind strongly to unmodified Bp and to HPMA-based copolymers containing multiple Bp chains covalently bound to the polymer backbone.[22] However, prior to the biological evaluation of our BTX-based drug delivery system, it was necessary to verify if chemical modification of BTX with copolymers containing reactive TT groups does not destroy the binding activity of the resulting polymer-BTX conjugate to Bp and/or scFv-Bp. At the same time, it was important to check if the Bp incorporated in the recombinant protein scFv-Bp did not lose its binding activity with BTX and polymer-BTX conjugates by performing SEC, ITC and DLS experiments.

3.3.1 Size-Exclusion Chromatography

The association behavior of the polymer conjugates containing BTX (P-Dbco-BTX and P-BTX-Dox) with recombinant protein scFv-Bp was studied by SEC using MicroSuperose 12 column in aqueous PBS. First, we confirmed formation of the complex between unmodified BTX and recombinant protein scFv-Bp. The original peaks corresponding to BTX (t_R = 20 min) and scFv-Bp (t_R = 17.5 min) disappeared upon mixing of equimolar amounts of the two components and a new peak corresponding to a higher molecular weight adduct was observed (**Figure 1**). The surprisingly short retention time of the adduct (t_R = 12 min) indicates the formation of stable high-molecular weight associates with quite narrow molecular weight distribution, however, with molecular weight exceeding 10^6. Nevertheless, this result clearly demonstrated that the Bp sequence incorporated in the structure of recombinant protein scFv-Bp retained its binding affinity to BTX. The molecular characteristics of the copolymers and non-covalent complexes are summarized in **Table 1**.

Table 1. Basic characteristics of the copolymers

Copolymer	M_w [a]	M_w/M_n [a]	TT mol% [b]	Dox wt% [c]
P-TT[d]	27 200	1.03	11.6	0
P-TT-biotin-Dbco[d]	56 000	1.10	5.5	0
P-Dbco-BTX	87 000	1.14	0	0
P-Dbco-BTX/scFv-Bp	145 000	3.15	0	0
P-BTX-Dox	400 000	1.46	0	7.7
P-BTX-Dox/scFv-Bp	500 000	1.54	0	4.6

P-Dbco-scFv	207 000	1.95	0	
P-scFv-Dox	>1 000 000	>3.0	0	9.1
P-Dox	79 000	1.08		10.6
BTX/scFv-Bp	>1 000 000	1.45		

[a] Molecular weight determined by SEC in PBS using RI and LS detection.

[b] TT determined by UV/VIS spectrophotometry in methanol (ε_{305} = 10 300 L·mol^{-1}·cm^{-1}).

[c] Dox determined by UV/VIS spectrophotometry in methanol (ε_{488} = 8 200 L·mol^{-1}·cm^{-1}).

[d] SEC measurement in 80 % methanol 20 % 0.3 M sodium acetate, pH 6.5.

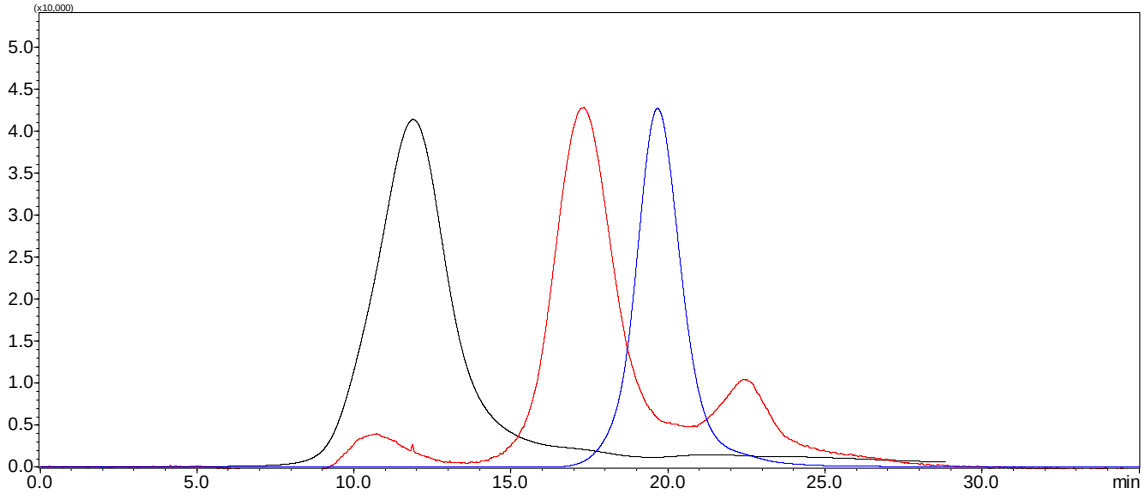


Figure 1. SEC chromatograms of BTX (blue), scFv-Bp (red) and equimolar mixture of scFv-Bp + BTX (black). MicroSuperose 12 column, eluent PBS, UV detector 280 nm, flow rate 0.1 mL/min and sample concentration of 3 mg/mL was used.

After the injection of the polymer-BTX conjugate P-Dbco-BTX (t_R = 16.5 min) and its equimolar mixture (molar ratio BTX/Bp = 1/1) with scFv-Bp, the formation of the non-covalent complex between the polymer and the protein was proved by a new broad peak with a maximum

around 15 min (**Figure 2**). The relatively wide molecular weight distribution (M_w/M_n = 3.15) is probably caused by statistical distribution of the various BTX molecules per individual polymer chain resulting in a variable number of scFv-Bp molecules per polymer. The formation of the polymer-protein complex clearly demonstrated that the binding affinity of BTX in the polymer conjugate P-Dbco-BTX was preserved.

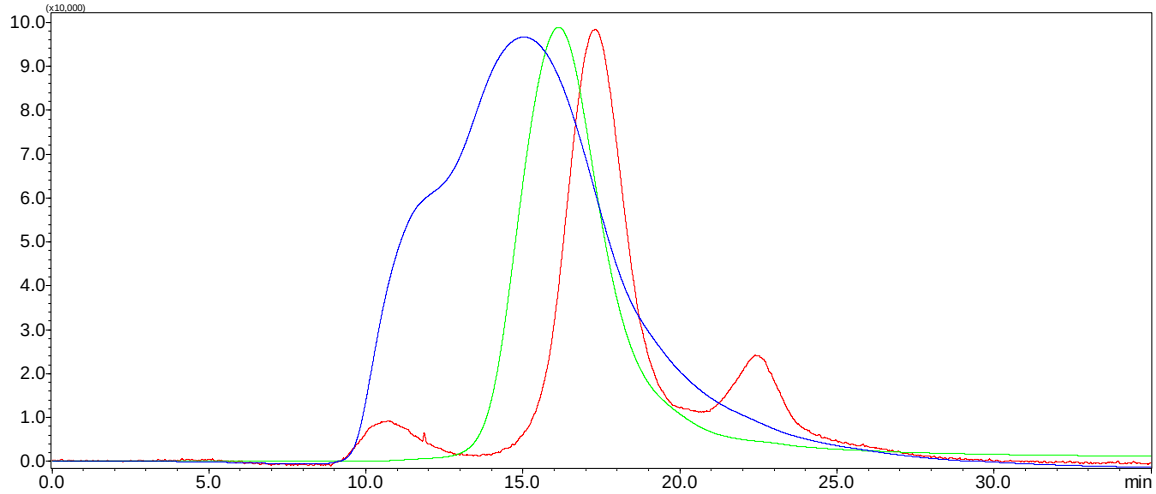


Figure 2. SEC chromatograms of scFv-Bp (red), P-Dbco-BTX (green) and equimolar mixture of the two components (blue). MicroSuperose 12 column, eluent PBS, UV detector 280 nm, flow rate 0.1 mL/min and sample concentration of 3 mg/mL was used.

Finally, an analogous experiment with polymer-BTX conjugate P-BTX-Dox containing the anticancer drug doxorubicin was performed. In this case, the addition of an equimolar amount of scFv-Bp to the polymer resulted in the disappearance of the protein peak; however, only a negligible shift of the newly formed peak compared with the peak of P-BTX-Dox was observed. A shoulder on the peak corresponding to unbound scFv-Bp appeared only after addition of 4-fold excess of the protein to the polymer (**Figure 3**).

This may be explained with the dramatic decrease of hydrophobicity of the original polymer-Dox conjugate upon complexation with the hydrophilic scFv protein. Most probably, the P-BTX-

Dox formed micellar structures in the aqueous media, which disassembled after the complexation with the scFv protein. The apparent weight-average molecular weight M_w = 400 000 of the original P-BTX-Dox increased to M_w = 500 000 after the addition of scFv-Bp, also indicating formation of the complex. The large difference between the measured M_w of the polymer precursor P-TT and the corresponding polymer conjugate P-BTX-Dox can be attributed to self-aggregation of the hydrophobic polymer conjugate in aqueous medium. While the SEC measurements of the polymer conjugates in methanol as a mobile phase provide molecular parameters of individual macromolecules, SEC in aqueous solution represents a more realistic result, reflecting their behavior in a physiologically relevant medium.

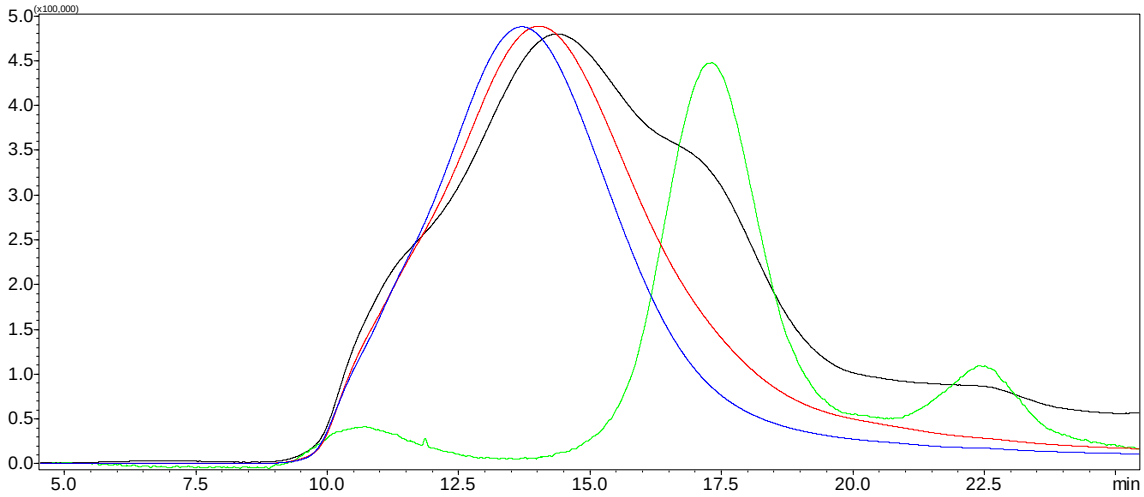


Figure 3. SEC chromatograms of scFv-Bp (green), P-BTX-Dox (blue), P-BTX-Dox + scFv-Bp (red, equimolar mixture) and P-BTX-Dox + 4×scFv-Bp (black, 4-fold excess of scFv-Bp). MicroSuperose 12 column, eluent PBS, UV detector 280 nm, flow rate 0.1 mL/min and sample concentration of 3 mg/mL was used.

In comparison with the non-covalent polymer-scFv complexes, covalent conjugate P-scFv-Dox has a higher molecular weight and dispersity resulting from the reaction of the multiple amino groups of the protein with the multiple reactive TT groups of the polymer. Consequently, the

non-covalent polymer-scFv complex containing Dox seems to be a good candidate for further investigation.

3.3.2 Dynamic Light Scattering

The conclusions from the previous section were further supported by DLS experiments conducted for individual components and their mixture (**Figure 4**).

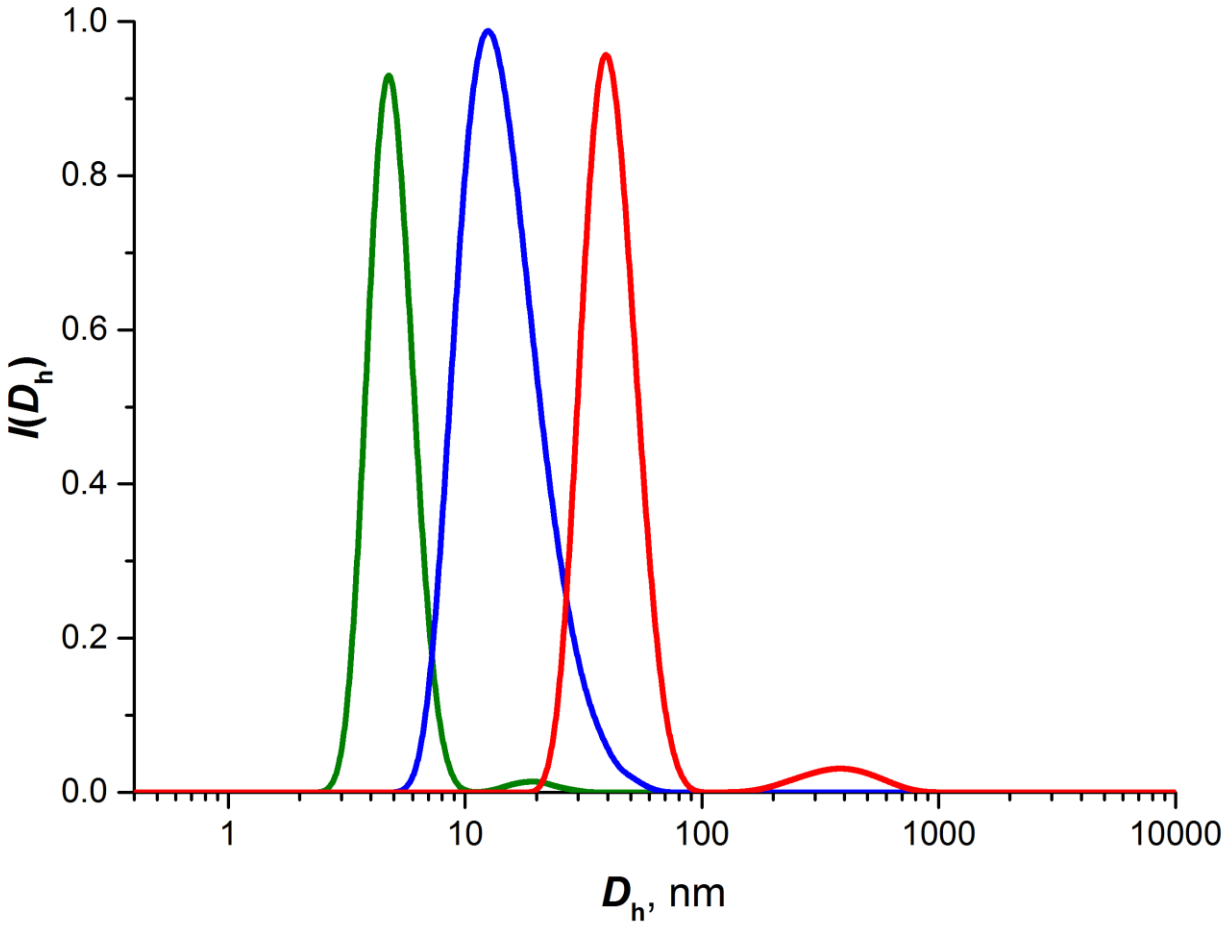


Figure 4. Volume weighted distribution function over hydrodynamic diameter Dh of scFv-Bp (green), P-BTX-Dox (blue), P-BTX-Dox + scFv-Bp (red, 2-fold excess of P-BTX-Dox).

The distribution function of the scFv-Bp protein manifests in the presence of a dominant peak corresponding to 5 nm that could be attributed to molecular dissolved proteins. The minor peak is related to a small fraction of protein aggregates that are also visible on the SEC chromatogram

(**Figure 3**). In contrast, the inspection of the distribution function for the polymer conjugate P-BTX-Dox reveals a single mode with a hydrodynamic diameter D_h of 20 nm. Keeping in mind that the molecular weight of the polymer precursor P-TT measured in 80% methanol (preventing aggregation) is 27 000 g/mol, this mode could be assigned to the aggregates of polymer conjugate, since the estimated hydrodynamic diameter D_h for a single chain of such molecular weight should in the range of 5–7 nm. Thus, the DLS results corroborate the SEC data, suggesting self-aggregation of the hydrophobic polymer conjugate in PBS. Complex formation between P-BTX-Dox and scFv-Bp protein is clearly visible on the distribution curve (**Figure 4,** red line); a bimodal distribution with a left peak corresponding to the particles with hydrodynamic diameter of 40 nm. This is indicative of complexation and subsequent association of the protein and the polymer conjugate. No peak related to individual protein or conjugate is manifested on the distribution function. The second peak might be attributed to a very small amount of large aggregates.

3.3.3 Isothermal Titration Calorimetry

To quantify the strength of binding of BTX or its polymer conjugate to the binding peptide Bp or scFv-Bp protein, ITC experiments were performed. ITC provided complete thermodynamic parameters of the binding constant (K_b), stoichiometry (N), enthalpy change of binding (ΔH), and entropy change of binding (ΔS) from the thermograms produced after integration of each titration followed by subtraction of the dilution heat. Gibbs free energies of each reaction were then calculated using the enthalpy and entropy results. Thus, ITC offered a direct tool for characterizing the thermodynamic properties of a binding process. The binding of BTX or its polymer conjugate to the binding peptide or scFv-Bp protein was studied by ITC in phosphate buffer solution at 37°C. The integrated heats of BTX or its polymer conjugate per mole of injectant is shown in **Figure 5**.

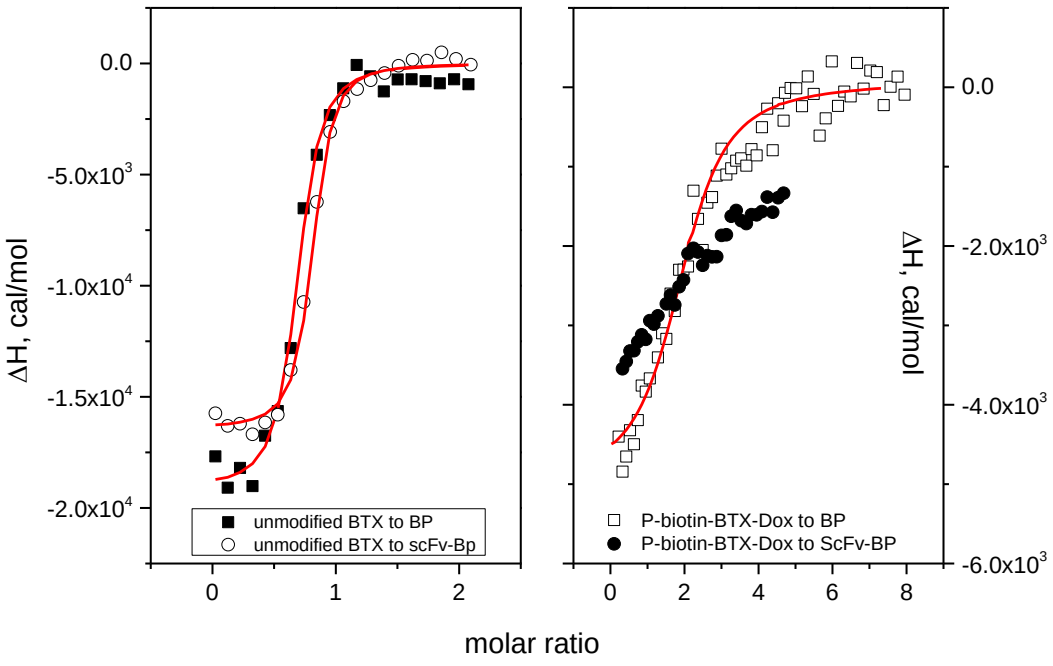


Figure 5. Calorimetric titration curves obtained for titration of 10 μM of BTX or its BTX-polymer conjugate solution into 1μM binding peptide or scFv-Bp protein in PBS solution at 37°C.

The results proved the complexation of BTX or its BTX-polymer conjugate with the binding peptide Bp or scFv-Bp protein. It is exothermic process and the thermodynamic parameters calculated using one-set-of-sites model are listed in Table 2. On the basis of the thermodynamic data, it is concluded that the complexation of BTX or BTX-polymer conjugate with Bp or scFv-Bp is enthalpy driven, not entropy driven. Indeed, both values of ΔH and TΔS are negative making the -TΔS term in the Gibbs equation positive. The values of stoichiometry for the complexation of BTX with binding peptide and scFv-Bp protein are 0.66 and 0.78, and the binding constant (K_b) of 7.3 and 12.2×10^6 mol^{-1}, respectively. The stoichiometry value lower than 1 is often due to inherent aggregation of proteins, which are easily detectable as shown in **Figure 1, 2,** and **3** for the SEC data. A general thermodynamic profile for the binding of pure BTX to binding peptide or scFv-Bp protein is relatively similar. The overall discrepancy in ΔH, TΔS, N, and K_b values could be explained by small differences in the binding site between the binding peptide Bp or protein scFv-Bp. In contrast, the covalent binding of BTX to the pHPMA main chain has a significant influence on a binding event. Although the ΔH values for BTX and P-BTX-Dox conjugate are only two-fold different for the same type of peptide, indicating that complexation of P-BTX-Dox with protein is energetically a less favorable process, the binding constant for P-BTX-Dox conjugate is one order of magnitude lower in comparison with pure BTX (**Table 2**). The possible explanation for such discrepancy is decreased accessibility of BTX moiety due to the steric hindrance caused by the polymer. Another peculiar observation for the

binding of P-BTX-Dox conjugate is the shift of stoichiometry to the values above 2, as evidenced by the SEC and DLS data where large aggregates of P-BTX-Dox conjugate were observed in solution.

Table 2. Thermodynamic parameters for the complexation of BTX or polymer-BTX conjugate with binding peptide Bp or scFv protein in PBS at 37°C

Ligand	Substrate	ΔH, kcal/mol	TΔS, kcal/mol	N, sites	K_b, 10^6 mol^{-1}
BTX	Bp	-19.1±0.4	-9.3	0.66±0.01	7.3±1.6
	scFv-Bp	-16.4±0.5	-6.4	0.78±0.02	12.2±0.5
P-BTX-Dox	Bp	-11.7±0.6	-3.1	2.1±0.2	0.5±0.3
	scFv-Bp	-4.3±0.5	-3.7	2.1±0.1	1.4±0.4

Taken together, the ITC experiments explicitly provide evidence of complexation of BTX to binding peptide or scFv-Bp protein regardless of its existing in solution as a single moiety or bound to a pHPMA chain. The binding was observed in both cases, although these binding processes are different in some features.

3.4 Cell Binding Studies of GD2-Targeted Polymer-Drug Conjugates

The aim of this study was to design, synthesize and evaluate polymer-Dox conjugates targeted against GD2-positive tumor cells, murine T-cell lymphoma EL4[23] and human neuroblastoma UKF-NB3.[5] Murine fibroblast cells NIH 3T3 were used as a negative control. Two different anti-GD2-targeted polymer-Dox systems were compared: a) covalent conjugate P-scFv-Dox prepared via a repeatedly described[24,25] and often used aminolytic reaction between the reactive TT groups

of the polymer with the primary lysine residues of scFv of anti-GD2 monoclonal antibody, and b) non-covalent complex P-BTX-Dox / scFv-Bp between polymer-bungarotoxin-Dox conjugate and a recombinant protein consisting of scFv and bungarotoxin-binding peptide. It was hypothesized that the latter system P-BTX-Dox / scFv-Bp would exhibit superior binding activity and, consequently, also cytotoxicity against GD2-positive cell lines due to its better-defined structure compared with the covalent conjugate.

In the flow cytometry experiments, anti-His/FITC was used for detection of the recombinant scFv protein (containing His tag) on the cells, streptavidin/FITC detected presence of the biotinylated polymer on the cells. If the both probes provided a strong positive signal a conclusion could be made that both components of the polymer-scFv complex were bound to the cells.

Although the exact number and positions of the bonds between the polymer and the protein in the covalent conjugate P-scFv-Dox are not known and the resulting polymer-protein conjugate is probably a complex mixture of macromolecules, the product still exhibited specific binding to both GD2-positive cell lines as demonstrated by flow cytometry (**Figure 6**). No binding to GD2-negative cell line NIH 3T3 was observed (**Figure S1A**, Supporting Information).

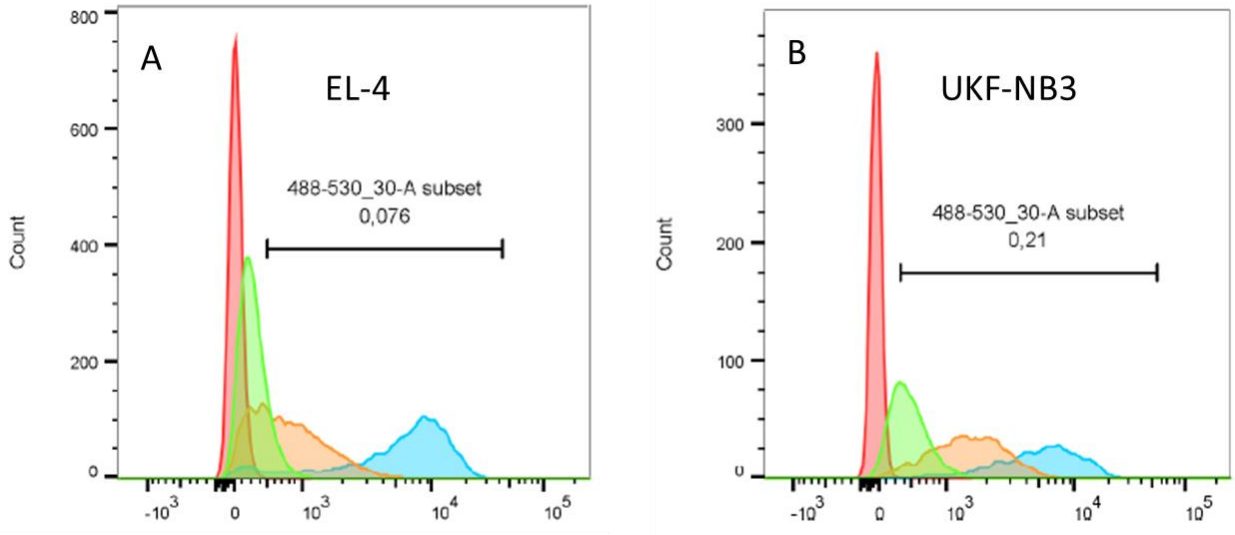


Figure 6. Flow cytometry analysis of (A) EL-4 and (B) UKF-NB3 cell binding by the targeted covalent conjugate P-scFv-Dox. Along the x-axis, the histograms show fluorescence intensity of the cells alone (red), cells incubated with scFv and anti-His-FITC (blue), cells incubated with P-scFv-Dox and anti-His-FITC (orange) and cells incubated with P-scFv-Dox and streptavidin-FITC (green).

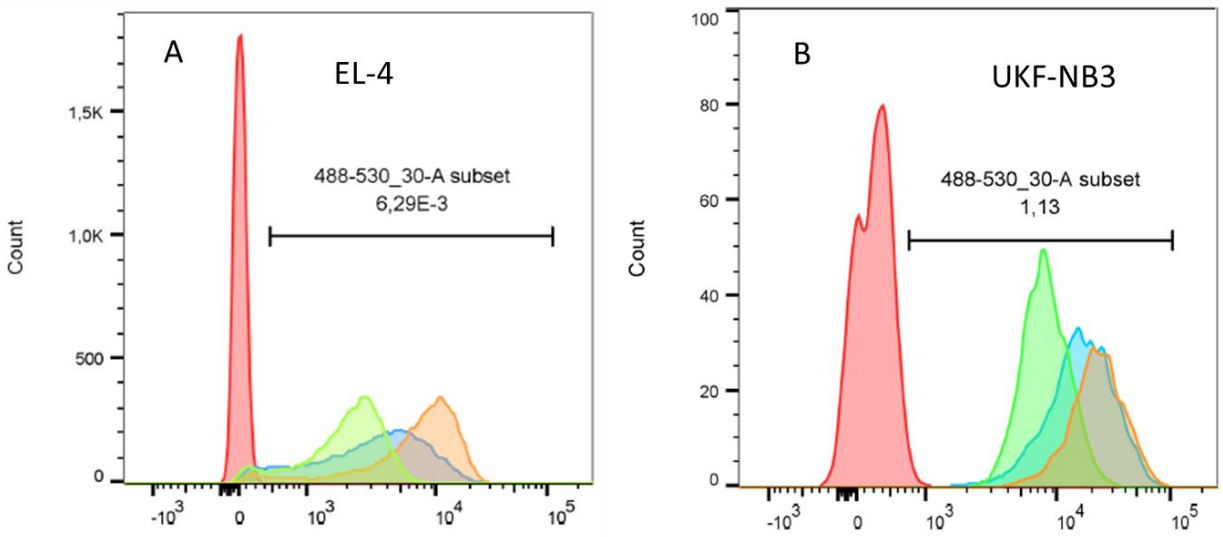


Figure 7. Flow cytometry analysis of (A) EL-4 and (B) UKF-NB3 cell binding by the targeted non-covalent complex P-BTX-Dox / scFv-Bp. Along the x-axis, the histograms show

fluorescence intensity of the cells alone (red), cells incubated with scFv and anti-His-FITC (blue), cells incubated with P-BTX-Dox / scFv-Bp and anti-His-FITC (orange) and cells incubated with P-BTX-Dox / scFv-Bp and streptavidin-FITC (green).

The non-covalent complex P-BTX-Dox / scFv-Bp exhibits somewhat better binding activity against the GD2 positive cell lines compared with the covalent conjugate P-BTX-Dox, as indicated by the fluorescence intensity of the cells incubated with the targeted polymer conjugates and visualized with streptavidin-FITC (**Figure 7**, green histograms). Surprisingly, the cells treated with P-BTX-Dox / scFv-Bp and detected with anti-His-FITC (orange histograms) exhibited even higher fluorescence intensity than the cells incubated with scFv alone (blue histograms). This may be attributed to the presence of multiple scFv ligands attached to one P-BTX-Dox conjugate (containing in average about 4 BTX binding sites) as well as the less destructive character of the non-covalent scFv binding to the polymer compared to the covalent aminolytic approach.

Interestingly, the enhanced targeting ability, which is directly connected with the increased cytotoxicity, was also observable from the cell counts during the cell binding experiment. The number of the living cells was substantially decreased after the incubation of the GD2-positive cells with the scFv-targeted samples. When the cell binding experiment was performed with a higher concentration (130 μg mL^{-1} Dox equivalent) of the targeted non-covalent complex, it led to a significant cytotoxic effect resulting in a very low number of living cells (using both GD2-positive cell lines) compared with the untreated control (**Figure S2**, **Table S1**, Supporting Information). No such toxicity was observed against the GD-2 negative control cell line NIH 3T3 (**Figure S1B**, **Table S1**, Supporting Information).

To further confirm the GD2 specificity of our targeted polymer complex, we prepared a mock recombinant protein scFv-Bp-B1 targeted against murine BCL1 leukemia as a negative control. We did not observe any cell binding neither with the GD2-negative cell line nor with GD2-positive cells using a P-BTX / scFv complex containing the irrelevant scFv-Bp-B1 (**Figure S3B**, Supporting Info). No binding was observed with P-BTX alone either. The addition of the polymer complex with the mock scFv to the cells also did not lead to the GD2-specific cytotoxicity described above.

3.5 Cytotoxic Activity of the Polymer Conjugates In Vitro

Cytotoxic activity of the polymer conjugates is evaluated using two GD2-positive tumor cell lines (one murine and one human) and compared with the activity of the non-targeted polymer-Dox conjugates and free Dox. The cytostatic activity is expressed as IC_{50} value corresponding to the concentration of the Dox equivalent in the sample resulting in 50 % of dead cells. The results of the cytotoxicity study against murine T-cell lymphoma EL4 are summarized in **Table 3** and clearly show that while the IC_{50} values of the non-targeted polymer-Dox conjugates P-Dox and P-BTX-Dox are approximately the same (ca 5 μg mL^{-1} of Dox equivalent), the non-covalent targeted polymer-protein complex P-BTX-Dox / scFv-Bp exhibits approximately 10× higher cytotoxicity (0.53 μg mL^{-1}) compared with the non-targeted conjugates. Using the other GD2-positive cell line (human neuroblastoma UKF-NB3), the increase in cytotoxicity of the targeted non-covalent complex compared with the covalent conjugate was only 3-fold, from 0.37 μg mL^{-1} to 0.14 μg mL^{-1}. Nevertheless, the difference clearly shows the advantages of the non-covalent approach over the traditional covalent conjugation methods.

Surprisingly, the covalent targeted polymer conjugate P-scFv-Dox had almost the same cytotoxicity (5.7 μg mL^{-1}) as the non-targeted conjugates, although flow cytometry proved that it

retained some antigen binding affinity. This result supports our hypothesis regarding superiority of the non-covalent targeting approach over the aminolytic conjugation of the protein ligands to polymer therapeutics.

Table 3. Cytostatic activity of the scFv-targeted and non-targeted polymer−Dox conjugates and free Dox against EL4 T-cell lymphoma and UKF-NB3 neuroblastoma.

Sample	EL4 cells	UKF-NB3 cells
P-Dox	5.0 ± 0.4	0.25 ± 0.03
P-scFv-Dox	5.7 ± 0.7	0.34 ± 0.02
P-BTX-Dox	5.2 ± 0.5	0.37 ± 0.03
P-BTX-Dox / scFv-Bp	0.53 ± 0.09	0.14 ± 0.01
Dox	0.02±0.01	0.007±0.002

[a] IC_{50} (+/- SD) / μg mL^{-1}, concentration of Dox equivalent in the sample inhibiting growth of the 50% cells compared with the untreated control.

4. CONCLUSIONS

In recent years, numerous hybrid systems for biomedical applications based on the combination of a synthetic polymer and a recombinant protein have been reported. Nevertheless, the need for new methods enabling conjugation of proteins to polymer carriers is very urgent, especially in the field of targeted polymer therapeutics and diagnostics. This study presented a new non-covalent anchoring system based on strong interaction between bungarotoxin and bungarotoxin-binding peptide for attachment of a targeting recombinant protein to a polymer-drug conjugate. This system allows the non-destructive and site-specific binding of a recombinant single chain antibody fragment of anti-GD2 antibody to polymer-drug conjugates thus enabling the highly-efficient cell binding and cytotoxicity against cells of GD2-positive

tumors. The new targeted polymer system is superior to the traditional covalent polymer-protein conjugate in terms of antigen-specific cell binding and cytotoxicity.

Cell viability/proliferation assays using GD2-positive cell lines (EL-4 and UKF-NB3) showed the strong advantage of the non-covalently bound targeting ligand over the aminolyticaly bound ligand. While the non-specifically bound scFv-polymer conjugate has IC_{50} similar to the non-targeted polymer, the scFv/polymer complex prepared via BTX-BTXbp interaction exhibited a 10× lower IC_{50} value against murine EL4 T-cell lymphoma and almost 3× lower IC_{50} against human neuroblastoma UKF-NB3.

In vivo evaluation of the anti-tumor efficacy of such new targeted polymer therapeutics using an animal model is ongoing. We believe that similar non-covalent anchoring systems could be successfully utilized in the design of new polymer therapeutics and diagnostics intended for clinical practice.

ASSOCIATED CONTENT

Supporting Information.

Additional flow cytometry data.

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Author Contributions

‡ R.P. and V.K. contributed to the manuscript equally.

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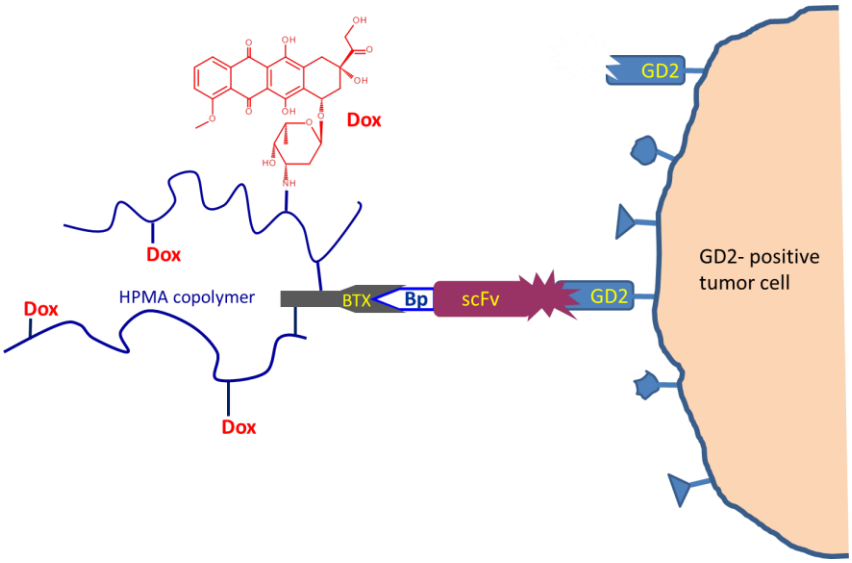
ToC graphic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