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RESEARCH PAPER

Multi-component Polymeric System for Tumour Cell-Specific Gene Delivery Using a Universal Bungarotoxin Linker

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ABSTRACT

Purpose A new universal tool for specific, non-covalent and non-destructive attachment of a recombinant antibody fragment to a polymer-modified adenovirus has been utilised to regulate the tropism of adenoviral gene delivery vector.

Methods We have prepared a multivalent reactive *N*-(2-hydroxypropyl)methacrylamide-based copolymer (PHPMA) bearing an α-bungarotoxin-binding peptide (BTXbp). The copolymer was used for covalent surface modification of adenoviral vectors (Ad). The α-bungarotoxin protein (BTX) has a nanomolar binding affinity for BTXbp, allowing non-covalent linkage of BTX fusion proteins. A single chain variable fragment of anti-PSMA antibody bearing BTX (scFv-BTX) binding to the prostate-specific membrane antigen (PSMA) was conjugated with the copolymer-coated adenovirus to enable specific infection of prostate cancer cells via PSMA receptors.

Results As shown by ELISA, the copolymer-coated virus exhibited much reduced binding to anti-Ad antibodies. Infection of PC-3 and LNCaP prostate cancer cells was ~100-fold less efficient with copolymer-coated Ad than with un-modified Ad. Conjugation of scFv-BTX with Ad-PHPMA-BTXbp led to 5–10-fold restoration of infection in PSMA-positive LNCaP cells. In PSMA-negative PC-3 cells, the conjugation of scFv-BTX with Ad-PHPMA-BTXbp gave no enhancement of infection.

Conclusions We have shown that the presented Ad-PHPMA-BTXbp/scFv-BTX system can be used as a universal tool for a receptor-specific virotherapy.

KEY WORDS adenovirus · bungarotoxin · hydrophilic polymers · scFv · tumour targeting

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ABBREVIATIONS

Ad	adenovirus
BCA	bicinchoninic acid
BTX	α-bungarotoxin
BTXbp	α-bungarotoxin-binding peptide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HINGS	heat-inactivated normal goat serum
HPMA	*N*-(2-hydroxypropyl)methacrylamide
IPTG	isopropyl β-D-1-thiogalactopyranoside
Ma-GG-OH	*N*-methacryloylglycylglycine
Ma-GG-TT	3-(*N*-methacryloylglycylglycyl) thiazolidine-2-thione
PEG	poly(ethylene glycol)
PHPMA	*N*-(2-hydroxypropyl)methacrylamide-based copolymer
PBS	phosphate buffered saline
PSMA	prostate specific membrane antigen
scFv-BTX	single chain variable fragment of anti-PSMA antibody bearing BTX
TMB	3,3′,5,5′-tetramethylbenzidine

INTRODUCTION

Adenovirus-based gene therapy is a very promising approach for treatment of oncological diseases (1,2). The transduction efficacy of adenoviruses is still several orders of magnitude higher than that of any non-viral vectors. However, clinical application of adenovirus vectors to treat metastatic disease has been so far compromised by the poor target cell selectivity, high immunogenicity and rapid neutralisation by antibodies and clearance of the vectors from the bloodstream (3–6).

It has been demonstrated that modification of adenoviruses with hydrophilic synthetic polymers based on poly(ethylene glycol) (PEG) (7) or *N*-(2-hydroxypropyl) methacrylamide copolymer (PHPMA) (8,9) significantly prolongs plasma circulation times and ablates non-specific cell entry. In addition, surface modification of the adenovirus capsid with multivalent polymers provides a platform for retargeting with receptor-specific ligands, such as growth factors (10), tumour-specific peptides (11) or folates (12). However, the covalent attachment of targeting ligand to the coating polymer suffers from several limitations. First, the reaction conditions required lead to at least partial loss of the biological activity and specificity of the ligand. The resulting organisation of the polymer-ligand conjugate is usually not a chemically homogenous structure but rather a heterogeneous mixture of substances with various degrees of modification of the targeting molecule. Second, such modification does not necessarily present the ligand in the most effective orientation for subsequent receptor binding. Third, the use of amine reactive groups of the HPMA copolymer for retargeting can diminish the efficiency with which the polymer coats the adenovirus.

With regards to potential future applications and regulatory approval of the new targeted therapeutics based on polymer-coated adenoviruses, a more specific technique of conjugating the targeting moiety to the vector to provide better defined structures is desirable. A specific, strong, non-covalent interaction between two peptides or proteins represents a possible solution to this requirement.

In this paper, we introduce a new universal tool for specific, high affinity and non-destructive attachment of recombinant proteins to other macromolecules such as synthetic or natural polymers or proteins. The principle of the method is based on the strong and non-covalent interaction between acetylcholine receptors and α-bungarotoxin (BTX).

It has been reported in literature (13,14) that synthetic peptide with amino acid sequence GSGGSGGTGYRS WRYYESSLEPYPD (BTXbp) derived from the structure of acetylcholine receptor binds BTX with high affinity, thus inhibiting its interactions with acetylcholine receptor with an IC_{50} of 2 nM. We linked the BTXbp to a reactive HPMA-based hydrophilic copolymer and used the resulting peptide-bearing copolymer (PHPMA-BTXbp) for surface modification of an adenoviral gene delivery vector. A recombinant anti-PSMA antibody scFv fragment containing the BTX binding region (scFv-BTX) was prepared and added to the polymer-modified BTXbp-bearing adenovirus (Ad-PHPMA-BTXbp) encoding luciferase as a reporter gene (Ad) as shown in Fig. 1. The fusion protein scFv-BTX served as a targeting ligand redirecting the natural viral tropism to prostate-specific membrane antigen (PSMA) on prostate cancer cell lines.

MATERIALS AND METHODS

Chemicals

Peptide BTXbp (GSGGSGGTGYRSWRYYESSLEPYPD, 2728 $gmol^{-1}$) was prepared by standard Fmoc solid phase peptide synthesis by peptide company Vidia (Vestec, Czech Republic). All other reagents and solvents were purchased from Sigma-Aldrich (Czech Republic). Methacryloyl chloride, 1-aminopropan-2-ol and dichloromethane were distilled immediately before use.

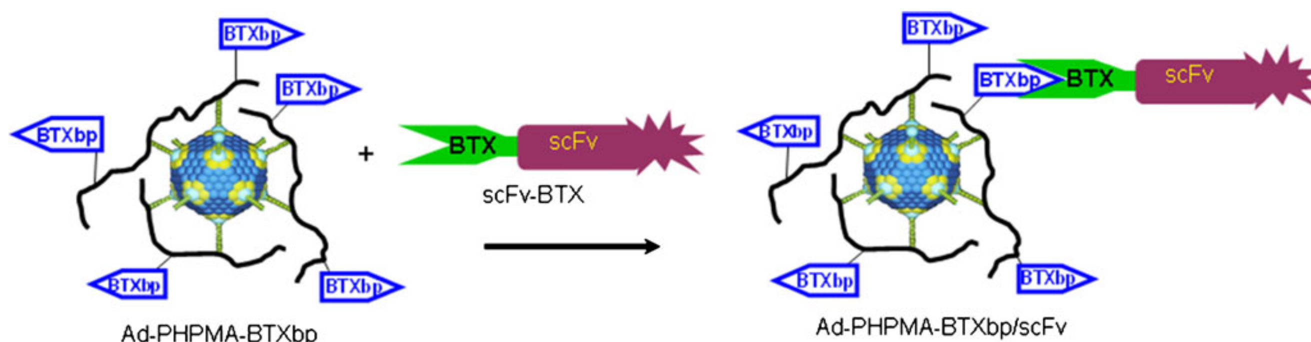


Fig. 1 Schematic representation of a polymer-modified adenovirus targeted with a recombinant scFv antibody fragment attached via a bungarotoxin linker.

Synthesis of Monomers

N-(2-hydroxypropyl)methacrylamide (HPMA) was prepared by reaction of methacryloyl chloride with 1-aminopropan-2-ol in dichloromethane (15). *N*-Methacryloylglycylglycine (Ma-GG-OH) was prepared by Schotten-Baumann acylation of glycylglycine with methacryloyl chloride in aqueous alkaline medium (16). 3-(*N*-Methacryloylglycylglycyl) thiazolidine-2-thione (Ma-GG-TT) was prepared by the reaction of Ma-GG-OH with 4,5-dihydrothiazole-2-thiol in *N*,*N*′-dimethylformamide in the presence of *N*,*N*′-dicyclohexylcarbodiimide (17).

Reactive Copolymer PHPMA

The copolymer poly(HPMA-*co*-Ma-GG-TT) was prepared by radical solution copolymerization of HPMA (90 mol %) and Ma-GG-TT (10 mol %) performed in dimethyl sulfoxide at 50°C for 6 h. The concentration of monomers in the copolymerization mixture was 15 wt % and that of 2,2′-azobisisobutyronitrile initiator was 2 wt % (16). For characteristics of resulting copolymer **1** see Table I.

Reactive Copolymer with BTXbp

The conjugation of BTX-peptide (20 mg, 7.3 μmol) to reactive copolymer **1** (78 mg, 41 μmol TT) was performed in 3% aqueous solution at constant pH 7.4. The reaction course was monitored using reversed-phase HPLC. The reaction was terminated after 1.5 h by addition of CH_3COOH (20 μl, 0.33 mmol). The polymer conjugate was purified by gel filtration on Sephadex G-25 in water (PD 10 column; Pharmacia) and freeze-dried, yielding 78 mg (80%) of reactive polymer-peptide conjugate **2**. For the characteristics, see Table I.

Analytical Methods

The monitoring of conjugation of the peptide to reactive copolymer was performed by HPLC using column Chromolith Performance RP-18e, 100×4.6 mm (Merck, Germany) and linear gradient water-acetonitrile, 0–100% acetonitrile in the presence of 0.1% TFA with a UV-VIS diode array detector (Shimadzu, Japan). Determination of molecular weights and polydispersity of the copolymers was carried out by size exclusion chromatography on a HPLC system (Shimadzu) equipped with refractive index, UV, and multiangle light-scattering DAWN 8 EOS (Wyatt Technology Corp., Santa Barbara, CA) detectors using Superose 6 column (Pharmacia) and 0.3 M acetate buffer, pH 6.5 at a flow rate of 0.5 ml/min. The calculation of molecular weights from the light-scattering detector was based on the known injected mass presuming 100% mass recovery. The content of TT groups was determined spectrophotometrically on a UV/VIS spectrophotometer Helios Alpha (Thermospectronic, UK) using the absorption coefficient for TT in methanol, $\varepsilon_{305}=10\ 280\ \mathrm{l{\cdot}mol^{-1}{\cdot}cm^{-1}}$.

Recombinant Protein Ligand scFv-BTX

To construct a PSMA-specific scFv-BTX fusion protein, the scFv J591 (a kind gift from M. Sadelain, New York, USA) was introduced into the pCES1 vector (18) via Sfi I and Not I sites next to the BTX sequence (Gene-synthesis by Baseclear, Leiden, The Netherlands), which was cloned via Not I and Bam HI. After verification of the correct sequence, the resulting pCES1-scFv-BTX vector was introduced into TG1 bacteria via electroporation (Biorad micropulser). One positive colony was then grown overnight in 50 ml of 2×TY medium containing ampicillin 100 μg/ml and glucose 2% at 30°C. The next day, 7.5 ml of the overnight culture was added to 750 ml of 2×TY medium with ampicillin 100 μg/ml and glucose 1% and grown for approximately 2 h until OD_{600} of 0.8–1.0 was reached. Protein production was then induced by the addition of 1 mM IPTG and overnight incubation at 30°C.

The next day, bacteria were spun down at 4,000 rpm for 15 min, and medium was stored for purification of the fusion protein.

The fusion protein was purified by FPLC using a His-trap crude column after adjustment of the immidazole concentration to 5 mM. Protein eluted in PBS (10 mM phosphate buffer saline) with 500 mM immidazole was desalted using Zebra desalting Spin columns (Thermo

Table I Basic Characteristics of the Reactive Copolymers

Polymer	Structure	M_w [a] g·mol^{-1}	M_w/M_n	Peptide [b] wt %	TT [c] mol %
1	p(HPMA-*co*-Ma-GG-TT)	32 000	1.9	–	8.2
2	p(HPMA-*co*-Ma-GG-BTXbp–*co*-Ma-GG-TT)	65 000	2.7	16.7	4.1

[a] Determined by SEC with light scattering detector

[b] Determined by reverse-phase HPLC

[c] Determined by UV/VIS spectrophotometry in methanol ($\varepsilon_{305}=10280\ \mathrm{lmol^{-1}\ cm^{-1}}$)

scientific). The final fusion protein preparation was checked for concentration by BCA protein assay kit (Pierce) purity and correct size by SDS-PAGE and western blotting.

Modification of the Adenoviruses with Polymers

LNCap and PC-3 carcinoma cells were obtained from ATCC; PSMA status was confirmed using an anti-PSMA antibody (AbCam) and flow cytometric analysis. E1, E3-deleted Ad5 expressing CMV IE promoter driven luciferase was purchased from Hybrid Systems Ltd. (Oxford, UK) and is denoted "Ad" throughout.

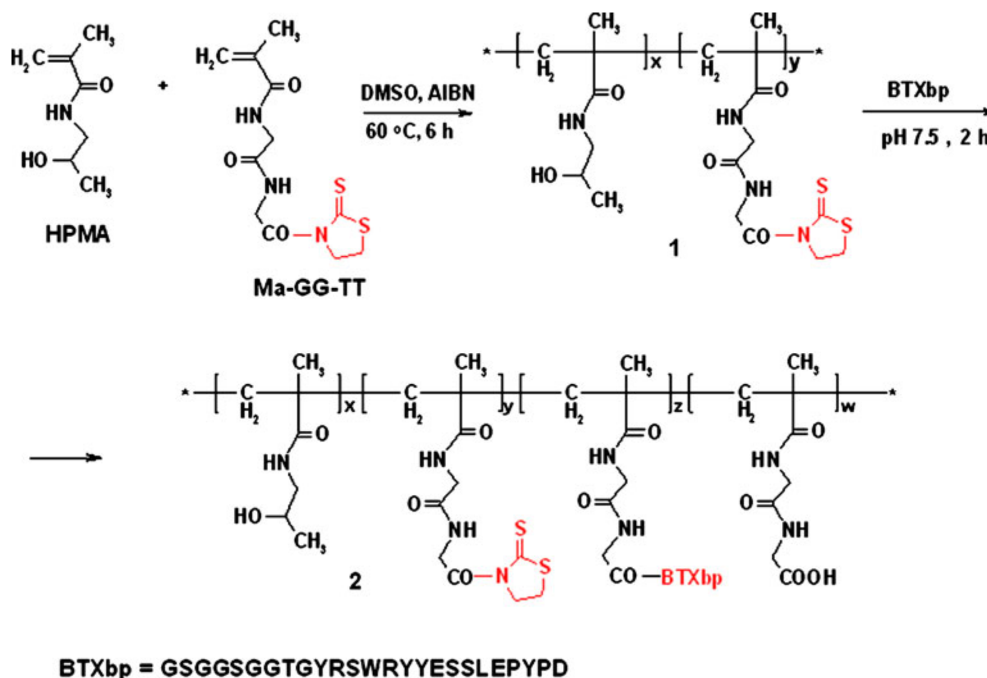
Polymer coating was performed by mixing Ad with PHPMA or PHPMA-BTXbp in 10 mM HEPES buffer (1 h, 10 mg/ml, pH 7.4) before purifying away un-reacted polymer using S400 columns 27-5140-01 (GE Healthcare). Recoveries were calculated using a Picogreen assay as previously reported for Ad-PHPMA using Oligreen (19). Mixing of 17µl (10^9 copies) of polymer-coated Ad with 0.05 mg/ml solution of scFv in a total volume of 60µl was then performed.

Anti-Ad Antibody ELISA to Assay Polymer Coating

Polyclonal rabbit anti-adenovirus antibody (AbCam, ab6982-100) was diluted 1/500 in coating buffer (Alpha Diagnostics International Inc. [ADI], 80050) and 50µl added to each well of a Nunc Maxisorb flat-bottom microplate (Fisher Scientific, 439454), and the plate was sealed (greiner Bio-one, 676001). After incubation overnight at 4°C, the plate was washed 10× with PBS using a Nunc Immuno Wash12. Fiftyµl of blocking buffer (coating buffer+5% HINGS) was added to each well, the plate sealed and incubated at 37°C for 1 h, then washed twice with PBS. Ad samples were diluted to 5×10^8 particles per well in diluent (PBS +5% HINGS) and 50µl added to each well. The samples were sealed and incubated overnight at 4°C before the plate was washed 10 times in PBS. Polyclonal goat anti-adenovirus-Biotin conjugate (AbD Serotec, 0151-9104) was diluted 1/100 in diluent, then 50µl was added to each well, sealed, and incubated at room temperature for 1 h. After washing 10× in PBS, 50µl of Avidin-HRP (eBioscience, 18-4100-94) diluted 1/1000 in diluent was added to each well and sealed and incubated at room temperature for 30 min. After washing 10× in PBS, 50µl of TMB substrate (ADI Inc, 80091) was added to each well and left to develop at room temperature for 10 min. Fiftyµl of stop solution (ADI Inc. 80100) was added to each well and the plate read at 450 nm, 1 s on Wallac Victor2 1420 multilabel counter. Data are represented as the Abs 450 nm value after subtraction of the value obtained for the negative control.

Cancer Cell Line Infections

Cells were plated (96 well plate, 10^4 per well) and 24 h later Ad samples (500 copies / cell) added. After 90 min, infection media were removed and washing in 150µl PBS performed



Scheme 1 Synthesis of the Reactive Copolymers

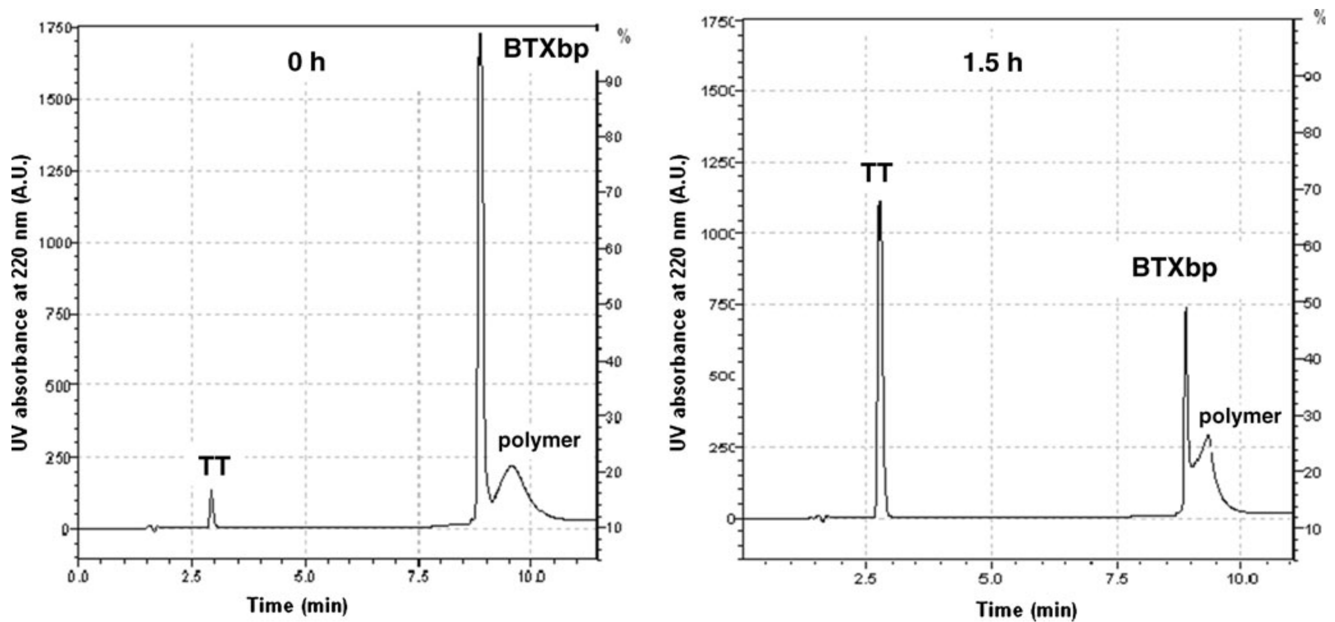


Fig. 2 HPLC chromatogram of the reaction mixture containing polymer **1** and BTXbp at the beginning of reaction and after 1.5 h using C18 column Chromolith, linear gradient water–acetonitrile, 0–100% acetonitrile, 0.1% TFA and UV detection at 220 nm.

before the addition of fresh media. Twenty-four h later, media were removed, washing in PBS performed, lysis solution was added and then luciferase levels measured using the luciferase reporter system E151A (Promega) and a luminometer (LB9507, Berthold). Uptake studies were performed in suspension by incubating 200,000 cells with 10^7 copies of Ad sample for 90 min with constant agitation. Cells were then spun (2,000 g, 5 min), supernatant removed and a PBS wash performed. After re-suspension in 400µl fresh media, half of the sample was analysed for Ad genome content by QPCR as in (5), and half the sample was re-plated and assayed at 24 h for luciferase expression.

RESULTS

Synthesis of the Polymer-Peptide Conjugates

Radical copolymerization of HPMA and Ma-GG-TT afforded reactive polymer precursor **1**. Polymer precursor **1** reacted with BTXbp in aqueous solution at constant pH 7.4 to yield the target reactive polymer-peptide conjugate **2** as shown in Scheme 1. The content of reactive TT groups decreased during 1.5 h of the reaction from 8.2 mol % to final 4.1 mol %. The amount of BTXbp bound to the polymer corresponded to 1.1 mol %; the remaining TT groups were hydrolyzed during the coupling reaction. The HPLC profile is shown in Fig. 2, and the molecular weights and polydispersity values of the polymer precursor and the polymer peptide conjugate are summarized in Table I.

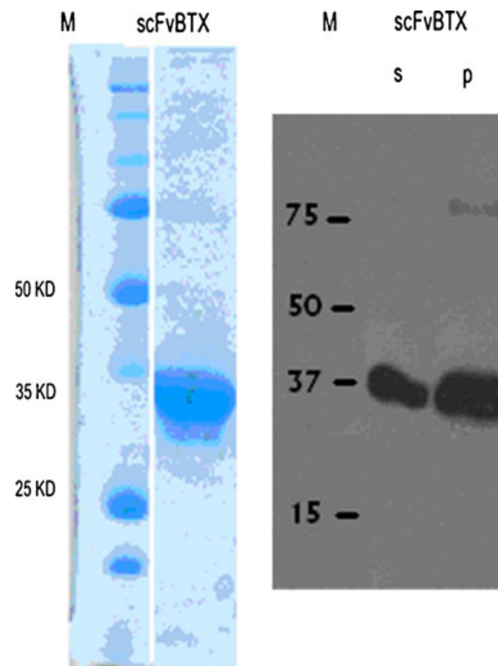


Fig. 3 Production and purification of the scFv-BTX fusion protein. The recombinant protein scFv J591-BTX was produced in E. Coli as described in Materials and Methods. The fusion protein was purified from medium using a His-trap crude column and analyzed by SDS-PAGE (left picture) and western blotting (right picture) for purity and correct size. By SDS-PAGE, a pre-dominant band of +/− 37 KD was obtained (>90% purity). This was confirmed by western blotting using anti-c Myc antibody 9E10 (s: non-purified medium, p: purified). M = molecular weight marker lane.

Recombinant Protein Ligand scFv-BTX

The recombinant PSMA-specific scFv-BTX fusion protein was cloned into the pCES1 vector and produced by standard protein expression in E-Coli, essentially as described in the Materials and Methods section. As shown in Fig. 3, the scFv-BTX protein was produced and could be purified (>90% purity).

Modification of Ad with PHPMA-BTXbp Polymers

The binding of anti-adenovirus antibodies to Ad-PHPMA-BTXbp was assessed by ELISA (see methods). Fig. 4 shows that antibody binding to Ad was higher than the level of binding to Ad-PHPMA-BTXbp or Ad-PHPMA. Indeed, the Abs 450 nm reading achieved with Ad-PHPMA-BTXbp or Ad-PHPMA was equivalent to the signal achieved using 1.25×10^8 copies of Ad, indicating the binding of antibodies had been reduced 75%. The level of antibody binding to Ad-PHPMA or Ad-PHPMA-BTXbp was equivalent. This indicates that the presence of the BTXbp does not diminish the ability of the PHPMA to coat and protect Ad.

Retargeting with scFv-BTX

Having verified the ability of the PHPMA-BTXbp to coat Ad, fluorimetry/SDS-PAGE analysis of the binding of Ad-PHPMA-BTXbp to BTX-Alexa488 confirmed that Ad-PHPMA-BTXbp could interact with BTX (data not shown). Infections in prostate cancer cell lines were then used to test whether retargeting of Ad-PHPMA-BTXbp could be achieved using scFv-BTX (Fig. 5). In PSMA-positive LNCaP cells (Panel A) luciferase transgene expression was knocked down ~100-fold upon coating Ad with either PHPMA-BTXbp or conventional PHPMA. However, after incorporation of the targeting scFv-BTX ligand, activity of Ad-PHPMA-BTXbp was restored between 5- and 10- fold ($p<0.001$), whereas the activity of Ad-PHPMA was not altered. In PSMA-negative PC-3 cells, infection activity was not rescued by the incorporation of the scFv-BTX targeting moiety (Panel B).

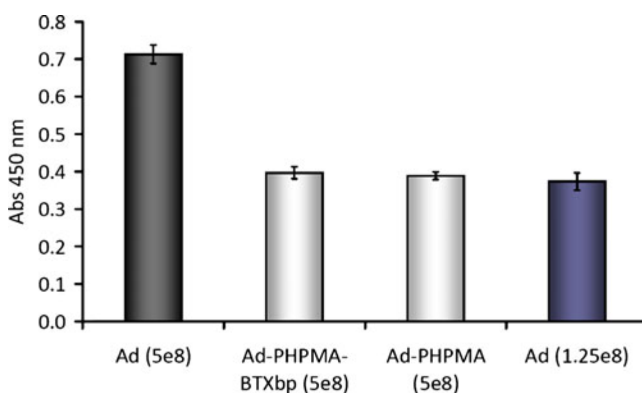


Fig. 4 PHPMA-BTXbp provides efficient coating of Ad. Ad, Ad-PHPMA-BTXbp or Ad-HPMA were exposed to an anti-Ad antibody-based sandwich ELISA (see methods). 5×10^8 or 1.25×10^8 copies per well, $N=4$, standard error shown.

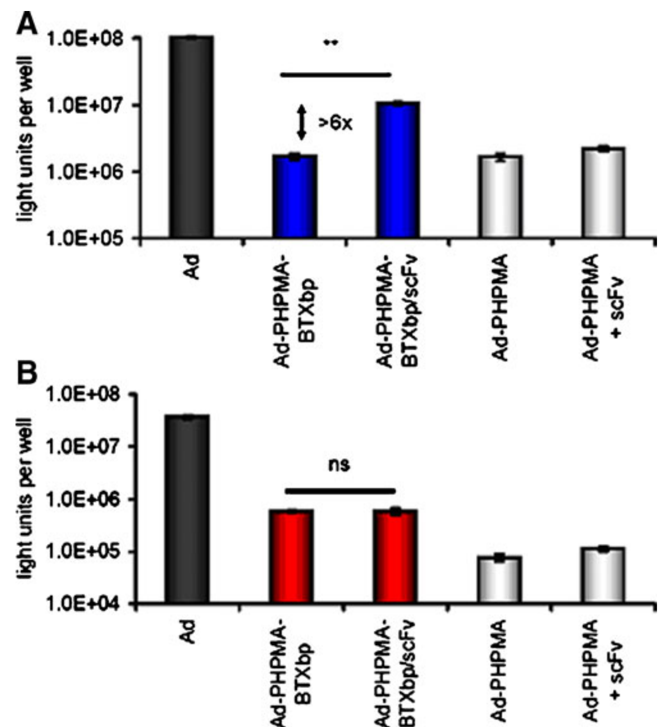


Fig. 5 Luciferase expression in PSMA-positive LNCap cells and PSMA-negative PC-3 cells after incubation with unmodified or polymer-modified Ad. Ad, polymer-coated Ad or polymer-coated, retargeted Ad was prepared as described in the Materials and Methods and used to infect LNCaP (panel A) or PC-3 cells (panel B). Luciferase reporter gene expression was assayed at 24 h (see Materials and Methods). $N=4$, standard deviation shown, $** = p<0.005$.

To verify that the presence of scFv-BTX instigated improved infection by enhancing the level of binding of Ad-PHPMA-BTXbp to cells, an association assay was performed and quantitative polymerase chain reaction (QPCR) used to measure the number of Ad genomes in the cells (see Materials and Methods). Fig. 6 demonstrates that, in LNCaP cells, coating of Ad with PHPMA-BTXbp leads to a decrease in cell association from 15% of the input dose to less than 5% (Panel A). However, incorporation of targeting scFv-BTX moiety leads to a significant ($p<0.05$) 2-fold restoration of cell association. It is notable that this small increase in cell association leads to a much greater increase in transgene expression (9-fold). In PSMA negative PC-3 cells, neither the cell association nor the resulting transgene expression was enhanced by the inclusion of scFv-BTX.

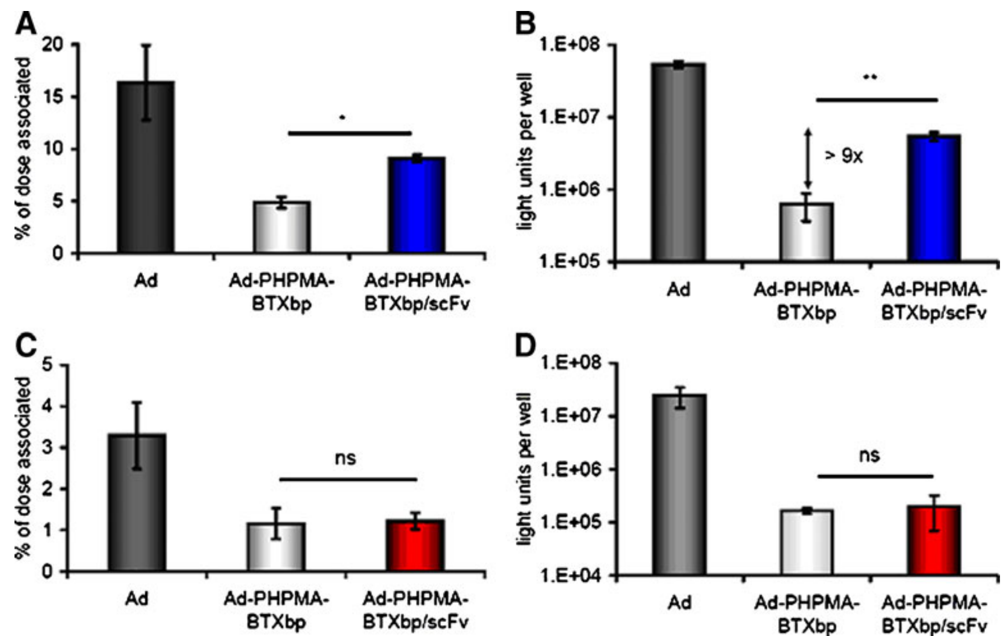


Fig. 6 Percentage of the dose of the vector associated with the cells after their incubation with unmodified or polymer-modified Ad. Sample was added to LNCaP (panels A and B) or PC-3 (panels C and D) cells in suspension, and the amount of Ad genomes associated with cells after 90 min was assayed by QPCR (see Materials and Methods). Half the sample was maintained to assay the resulting transgene expression. Data is represented as % of the input dose associated with cells. $N=3$, standard deviation shown, $* = p < 0.05$, $** = p < 0.005$.

DISCUSSION

Synthesis of the Polymer–Peptide Conjugates

Copolymers of HPMA with 4-nitrophenyl ester (ONp) or thiazolidine-2-thione (TT) derivatives of *N*-methacryloylated oligopeptides have been reported numerous times as reactive polymer precursors for synthesis of various biologically active compounds (BAC), such as cytostatics, peptides, enzymes and targeting antibodies. In most cases, the modification reaction between the polymer and BAC was based on the acylation of a primary amino group of BAC with reactive ester (ONp) or amide (TT) groups of the polymer. In the case of proteins or peptides containing more than one nucleophilic group susceptible to the acylation, the resulting polymer–peptide conjugate is at least partially branched. Consequently, its biological activity is compromised by the modification reaction. Careful choice of the reaction conditions is therefore crucial for minimization of these undesired reactions.

The advantage of using reactive polymer precursors with TT instead of ONp groups is their much slower rate of undesired hydrolysis in aqueous solutions. Here we report the use of this approach for attachment of BTXbp peptide to reactive copolymer **1** via the *N*-terminus of the peptide. This work was performed with the deprotected peptide due to the extreme difficulties with purification of the corresponding fully protected peptide derivative. Our initial attempts to bind the deprotected BTXbp to polymer **1** in organic solvent always led to highly branched or even insoluble cross-linked product, probably due to acylation of the side chain of the two Arg and four Tyr residues in the amino acid sequence of BTXbp. As the pK_a value of the guanidino group of arginine is 12.5, it remains protonated under physiological conditions and hardly available for the acylation at pH 7.4 used for the reaction. We also assumed that the eventual product of acylation of the phenolic group in tyrosine would be quickly hydrolyzed back as a result of general reactivity of phenyl esters with nucleophiles. Relative stability of the TT groups towards hydrolysis allowed us to bind most of the peptide to polymer in aqueous solution during 1.5 h and still preserve half of the TT groups for the subsequent modification of the adenovirus. The course of the binding reaction was monitored by HPLC (Fig. 2).

Nevertheless, the increase in polydispersity (from 1.9 to 2.7) of the polymer after the reaction with the peptide indicates that a very low degree of Arg modification on the guanidino group could not be ruled out completely.

Recombinant Protein Ligand scFv-BTX

The production of scFv-BTX fusion proteins in bacteria provides a fast and simple means to obtain retargeting ligands of choice. The method is easily expandable to other ligands, not limited to scFv, but also other antibody fragments or complete proteins. In addition to the PSMA-specific scFv J591 we have now also successfully expressed scFv-BTX fusion proteins specific for PSCA, HLA-A1/MAGE-A1, single domain antibody fragments (VHH) specific for EGFR, CEA and HLA-A2/multi-MAGE-A (data not shown).

Cell Transfection Studies

Successful systemic application of adenoviruses as gene delivery vectors is still associated with many serious hurdles, such as immunogenicity, low target selectivity and short blood circulation times of the vectors. Surface modification

of the viruses with hydrophilic synthetic polymers based, e.g., on PEG or HPMA is a possible promising strategy overcoming at least to some extent the mentioned obstacles (9).

The first step in changing the undesirable natural Ad tropism is detargeting of the virus from its natural cell entry mechanism via coxsackievirus and adenovirus receptor (CAR). In this study, this was achieved by surface coating of Ad with multivalent highly hydrophilic HPMA-based copolymers **1** or **2**. ELISA studies confirmed that high efficiency of coating and protection of the capsid had been achieved. The finding was further confirmed by the fact that in cancer cell line infection studies, the polymer modification of Ad caused a knock-down in transgene expression of more than 100-fold. We assume that this decrease of infectivity is a result of polymer-shielding of the knob domain of Ad which is responsible for CAR receptor-mediated cell entry. Moreover, efficient coating of the adenovirus with hydrophilic HPMA copolymer protects it from interaction with anti-adenovirus antibodies. This result is in accordance with previously published data showing significant decrease in immunogenicity of proteins after their modification with HPMA copolymers (by two orders of magnitude) (20,21).

The second important step is retargeting of the polymer-coated Ad to a specific cell receptor. Prostate-specific membrane antigen (PSMA) expressed on LNCaP cells was chosen in this work as a model target. To achieve PSMA targeting, a recombinant fusion protein scFv-BTX consisting of anti-PSMA antibody scFv fragment and BTX binding region was prepared. After the addition of scFv-BTX to Ad-PHPMA-BTXbp, the recombinant protein non-covalently bound to the BTXbp sequences on the polymer-coated virus forming PSMA-targeted viral gene delivery vector. This resulted in partial restoration of the luciferase transgene expression. Notably, no such restoration of luciferase expression was observed when scFv-BTX ligand was added to Ad-PHPMA with no BTXbp attachment handle. The results of a control experiment with PSMA-negative PC-3 cells showed no response to the addition of the targeting ligand, demonstrating the impressive selectivity and specificity of the system.

Qualitatively similar results were also obtained from a QPCR study designed to assess the degree to which the scFv-BTX influenced cell association. While the polymer-modified Ad without targeting ligand associated with cells >3 times less efficiently than unmodified Ad, the difference in the level of transgene expression of the modified *versus* unmodified viruses was almost 100-fold. This indicates that the polymer coating does not only protect the vector from a non-specific internalization but to a much larger extent compromises successful transgene expression. This emphasises the importance of the correct intracellular routing of the virus to its eventual activity. After retargeting of Ad-PHPMA-BTXbp with scFv-BTX ligand, the number of Ad genomes recovered from LNCaP cells increased just 2-fold. This increase instigated a significant 10-fold enhancement of reporter gene expression. These data indicate that in the case of non-specific cell entry of non-targeted polymer-modified Ad-PHPMA-BTXbp, a large proportion of the Ad is incapable of efficient transgene expression compared with Ad-PHPMA-BTXbp/scFv-BTX conjugate targeted to PSMA receptor. The fact that anti-PSMA retargeted Ad does not match the transgene expression obtained with unmodified Ad despite achieving similar cell association emphasises the improved efficiency of intracellular processing and routing of the Ad upon binding to its natural receptor (CAR) rather than PSMA. This indicates that although PSMA provides impressive selectivity of retargeting, for applications where higher efficiency is needed, alternate cell surface receptor targets may be preferable. This serves to highlight a great advantage of this system, in that an array of vectors and retargeting ligands can be tested using the same PHPMA-BTXbp molecule, and so the laborious direct conjugation of each new test ligand to PHPMA is not required.

CONCLUSIONS

We have prepared and characterized a multivalent reactive HPMA copolymer bearing an α-bungarotoxin-binding peptide (BTXbp). The polymer was used for covalent surface modification of adenoviral vectors containing luciferase reporter gene (Ad). The peptide BTXbp is known to have extremely high binding affinity to bungarotoxin (+/− 2 nM).

A recombinant protein consisting of a PSMA-specific antibody scFv fragment and the BTX binding region (scFv-BTX) was used for retargeting of the polymer-modified Ad to prostate-specific membrane antigen. While the polymer-modified Ad exhibited approximately 100-fold lower infectivity than the unmodified Ad, the addition of scFv-BTX targeting ligand to the polymer-coated Ad led to 5–10-fold restoration of luciferase expression in PSMA-positive LNCaP cells. No such increase of transduction activity was observed in PSMA-negative PC3 cells.

The data presented here demonstrate the impressive activity and flexibility of this novel PHPMA-BTXbp/scFv-BTX linker system that can provide a universal tool for achieving receptor-specific adenovirus gene transfer.

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