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RESEARCH PAPER

Hyaluronan Oligomers-HPMA Copolymer Conjugates for Targeting Paclitaxel to CD44-Overexpressing Ovarian Carcinoma

Gal Journo-Gershfeld • Dana Kapp • Yosi Shamay • Jindřich Kopeček • Ayelet David

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ABSTRACT

Purpose To evaluate the effect of the size of low molecular weight hyaluronan (LMW-HA) oligomers on the targeting ability of the HA-containing copolymers to the CD44-overexpressing cells for delivering Paclitaxel (PTX) to ovarian cancer.

Methods LMW-HA oligosaccharides of 4, 6, 8, 10, 12 and 14 sugar residues were attained by digestion of HMW-HA using hyaluronate lyase at different incubation times and then attached to FITC-labeled HPMA copolymer precursor. The binding and uptake of the HA-modified HPMA-copolymer into CD44-expressing cells was studied by flow cytometry and confocal microscopy. PTX was further attached to HPMA-copolymer precursor bearing HA oligosaccharide at the size of 34 monosaccharides, through an acid-sensitive hydrazone linker. The cytotoxicity of the polymer was tested using cell viability assay.

Results Polymer conjugates bearing HA oligomers at the size of 10 oligosaccharides and above (HA_{10-14}) bind actively and profoundly to CD44-overexpressing ovarian cancer cells (SK-OV-3) and internalize to the greatest extent relative to HA-polymer conjugates of 8 oligomers and below (HA_{4-8}). The HA-modified HPMA-copolymer PTX conjugate (P-$(HA)_{34}$-PTX) exhibited 50-times higher cytotoxicity towards CD44-overexpressing cells relative to the control, non-targeted, HPMA-copolymer PTX conjugate (P-PTX).

Conclusions P-$(HA)_{34}$-PTX was significantly more toxic than the non-targeted P-PTX in cells expressing high levels of CD44

KEY WORDS CD44 · HPMA copolymer-drug conjugates · hyaluronan · ovarian cancer · paclitaxel

ABBREVIATIONS

2AB	2-amino benzamide
AIBN	2,2′-azobis(isobutyronitrile)
BSA	bovine serum albumin
DCC	N,N'-dicyclohexyl-carbodiimide
DCU	N,N'-dicyclohexylurea
DMAP	4-dimethylaminopyridine
DOX	Doxorubicin
EPR	enhanced permeability and retention
FITC	fluorescein-5-isothiocyanate
GlcNAc	N-acetyl-D-glucosamine
GlcUA	D-glucuronic acid
HA	hyaluronic acid hyaluronan
HA-TBA	hyaluronic acid-tetrabutylammonium bisulfate
HMW-HA	high molecular weight HA
HPMA	N-(2-hydroxypropyl)methacrylamide
I	polydispersity
LEV	levulinic acid
LMW-HA	low molecular weight HA
MA-AP	N-(3-aminopropyl)methacrylamide
MA-AP(Boc)	N-(tert-butyloxycarbonyl-aminopropyl) methacrylamide

Gal Journo-Gershfeld and Dana Kapp contributed equally.

G. Journo-Gershfeld · D. Kapp · Y. Shamay · A. David (✉)
Department of Pharmacology, Faculty of Health Sciences, Ben-Gurion University of the Negev
Beer-Sheva 84105, Israel
e-mail: ayeletda@bgu.ac.il

J. Kopeček
Department of Pharmaceutics and Pharmaceutical Chemistry
University of Utah
Salt Lake City, Utah 84112-9452, USA

MA-AP-FITC	methacryloyl-aminopropyl-fluorescein-5-isothiocyanate
MA-GG-HZBoc	methacryloyl-glycylglycine hydrazide-Boc
MA-GG-OH	methacryloyl-glycylglycine
MA-GG-ONp	methacryloyl-glycylglycine p-nitrophenyl ester
Mn	number average molecular weight
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinium bromide
MW	molecular weight
Mw	weight average molecular weight
PFA	paraformaldehyde
PGA	Poly(L-glutamic acid)
PTX	Paclitaxel
RHAMM	receptor for hyaluronic acid-mediated motility
SEC	size-exclusion chromatography

INTRODUCTION

Paclitaxel (PTX) is an effective chemotherapeutic agent that is widely used for the treatment of several cancers, including breast, ovarian and non-small-cell lung cancer (1). Due to its high lipophilicity, PTX is difficult to administer and requires solubilization with Cremophor EL (polyethoxylated castor oil) and ethanol, which often lead to adverse side effects, including life-threatening anaphylaxis (2). In addition, it's very little aqueous solubility and the absence of selectivity for target tissue often results in a lower concentration of the drug that arrives at the tumor and causes severe side effects owing to the lack of specificity of the anticancer agent. To overcome these limitations, a variety of formulations and delivery systems are being investigated to administer PTX in a safer and more convenient manner (3–7). These formulations can be either passively or actively targeted into solid tumors. In passive drug targeting, the advantage lies in the accumulation of macromolecules, which penetrate the tumor due to the enhanced permeability and retention (EPR) effect that relies on the leaky tumor vasculature with its discontinuous endothelium, and on the ineffective tumor lymphatic drainage (8). For example, drug carrier systems based on the soluble synthetic polymer Poly-(L)-glutamic acid paclitaxel conjugates (PGA-PTX) (CT-2103) [OPAXIO™ formerly known as XYOTAX™] have been used in order to take advantage of the selective accumulation in tumors due to the EPR effect, and is currently under clinical developments as a single use agent or in combination with platinates in patients with relapsed ovarian and lung cancer (4). In active drug targeting, a specific targeting molecule could be combined with polymer conjugate to increase the uptake into the cells by receptor-mediated endocytosis. Several antigens and receptors, such as folate receptor (FR), tumor associated glycoprotein-72 (TAG-72), ovarian cancer-associated OA-3 antigen, and the hyaluronic acid (HA) receptors (CD44 and the receptor for HA-mediated motility (RHAMM)) have been reported to play an important role in ovarian cancer, some of them are targeted by growth factors, oligosaccharides, antibody fragments or other small molecules. For example, to develop ovarian cancer-targeted drug delivery systems, the OV-TL 16 monoclonal antibody which recognizes the OA-3 antigen (9), and HA which recognizes CD44 and RHAMM (10), have been previously reported as targeting ligands for drug-containing polymer conjugates.

CD44 is a cell-surface marker that is found at elevated levels in lymphoma, breast, colorectal, lung and ovarian carcinoma (11). This receptor binds HA, a negatively-charged, linear polysaccharide that is composed of repeating units of glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc) linked together via alternating β-1,4 and β-1,3 glycosidic bonds (12). Its molecular weight (MW) is diverse and can range from several hundred Daltons to as high as 10^7 Da. The high MWs of HA (HMW-HA) confers some unique viscoelastic and rheological properties and makes it suitable as a biomaterial for a variety of medical applications including shock-absorbing and lubricating component found in the cartilage and synovial fluid of the joint. Despite their relatively simple structure, HA oligomers have wide-ranging and even opposing biological functions depending on the size of the molecule. HMW-HA is anti-angiogenic, immunosuppressive, anti-inflammatory (13) and is important in embryonic development (14) tissue organization (15) and wound healing (16). Smaller HA oligomers (at the size of 50–100 monosaccharides) are known to be angiogenic (17), inflammatory, and immunostimulatory (13). The even smaller HA oligomers (12–28 monosaccharides) were shown to inhibit melanoma cell proliferation *in vitro* as well as formation of tumors from subcutaneously injected cells *in vivo* (18).

A variety of delivery systems containing HA as a targeting molecule for CD44 were created in order to increase intracellular concentrations of drug in tumor tissue. Liposomal systems were decorated with HMW-HA (19) and LMW-HA (20) oligomers on the surface for targeting DOX to tumor cells. Encapsulation of DOX in the HA-liposomes generated a significant reduction in the drug's IC_{50} values compared with free DOX in cell lines that overexpress HA receptors. Furthermore, polymeric systems such as poly(L-lysine) (PLL) (21,22) and poly(N-(2-hydroxypropyl)methacrylamide) (polyHPMA) (10) were grafted or conjugated with HA for the selective delivery of DNA and DOX, respectively, to CD44-overexpressing cells.

HMW-HA oligosaccharides (with MW higher than 500 kDa) are not readily soluble in water. Due to the high water binding capacity they form very quickly a viscous gel,

and the administration of such substances to the blood as a targeting ligand for drug delivery is not advantageous. Furthermore certain liver receptors specifically recognize HMW-HA and clear it rapidly from the systemic circulation (23). A possible way to circumvent this clearance is to use small, defined LMW-HA oligosaccharides (with MW lower than 10 kDa) that retain their targeting capabilities to CD44-overexpressing tumor cells. HA oligosaccharides, with different lengths and modified sequences, have been synthesized over the past decade (24), and thus can serve as ligand moiety for CD44-overexpressing cells.

In this work we evaluated the correlation between the lengths of LMW-HA oligomers and the binding affinity to the CD44 receptor, for targeting PTX to ovarian cancer, the cancer with the highest mortality rate among gynecological tumors. LMW-HA oligosaccharides of 4, 6, 8, 10, 12 and 14 sugar residues (750–2650 Da), henceforth referred to as HA_4, HA_6, HA_8, HA_{10}, HA_{12} and HA_{14}, were attained by digestion of HMW-HA (1×10^7 Da) using hyaluronate lyase at different incubation times and then attached to FITC-labeled HPMA copolymer precursor. The effect of the size of the targeting ligand on the binding and uptake by CD44-overexpressing ovarian (SK-OV-3) cells was studied. We further investigated the specific cytotoxicity of the HPMA copolymer-PTX conjugates bearing HA oligosaccharide at the size of 34 monosaccharides (HA_{34}, 6400 Da), P-(HA_{34})-PTX copolymer, towards cells with high and low level of CD44.

MATERIALS AND METHODS

Materials

All chemicals were of reagent grade and obtained from Sigma–Aldrich (Rehovot, Israel), unless mentioned otherwise. HA_{34} (MW=6400 Da) was purchased from LifeCore Biomedicals (MN, USA). N-(3-aminopropyl)methacrylamide (MA-AP) and N-(tert-butyloxycarbonyl-aminopropyl) methacrylamide (MA-AP(Boc)) were purchased from Polysciences, Inc (Warrington, PA). FITC was purchased from Fluka. p-nitrophenyl, tert.butyl carbazate, levulinic acid and o-phenylenediamine were purchased from Acros Organics (Yahud, Israel). Mouse anti-human CD44 antibody was purchased from BioLegend (San Diego, CA). PTX, >99.5% was purchased from LC laboratories (BAR-NAOR, Israel).

Cell Lines

Human ovarian cancer cells (SK-OV-3) were cultured in McCoy's 5A medium supplemented with 10% fetal calf serum, glutamine 2 mM, 1.5 g/l sodium bicarbonate and 1% antibiotics (penicillin, 10000 U/ml & streptomycin, 10 mg/ml). Human prostate cancer cells (DU-145) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine 2 mM, HEPES buffer 10 mM, pyruvate 1 mM and 1% antibiotics (penicillin, 10000 U/ml & streptomycin, 10 mg/ml).

Preparation and Characterization of LMW-HA Oligosaccharides

a) Enzymatic Degradation of HMW-HA

HMW-HA (1×10^7 Da) was enzymatically degraded by hyaluronate lyase according to a modified digestion protocol (25). Briefly, HA (sodium salt from streptococcus zooepidemicus) was dissolved in digest buffer (0.1 M sodium acetate, adjusted to pH 5.2 with glacial AcOH) at a concentration of 10 mg/ml and stirred at room temperature for 2 days. Hyaluronate lyase (streptomyces hyalurolyticus) was reconstituted in 20 mM sodium phosphate buffer with 77 mM sodium chloride, 0.01% bovine serum albumin (BSA), pH 7.0. 1000 U of the enzyme were added to the dissolved HA and were allowed to incubate at 37°C. Different incubation times (0.5, 1, 2, 4, 12 and 24 h) were required in order to receive different sized oligomers. The enzyme was deactivated by immersion in boiling water for 7 min. Afterwards, the solution was cooled and was then centrifuged at 17000 × g for 20 min at 4°C. The resultant pellet was discarded and the supernatant solution was lyophilized.

The lyophilized HA powder was dissolved in 0.5 ml of degassed 0.05 M ammonium bicarbonate buffer, filtered through a 0.45 μm filter and injected to a 16×100 cm XK-16/100 column packed with BioGel P-30, medium grade resin on a Fast Protein Liquid Chromatography (FPLC) system. Fractions (2 ml each) were collected and their absorbances were measured at 254 nm in the Ultrospec 2100pro UV/Visible spectrophotometer of the FPLC system. Peaks containing the HA oligomers were collected, lyophilized, re-dissolved in double-distilled water, and lyophilized again to remove residual ammonium bicarbonate salt.

b) Determination of HA Oligomer Size

The size of the HA oligomer was determined after the conjugation of each oligosaccharide fractions to 2-amino benzamide (2AB), a fluorescent labeling agent that labels glycans containing a free reducing terminus (26). The 2AB solution was prepared by dissolving 12.5 mg of the crystallized 2AB (λ_{ex}=330 nm, λ_{em}=420) in a 250 μl solution of acetic acid: dimethyl sulfoxide (AcOH:DMSO, 3:7). 15 mg of sodium cyanoborohydride ($NaCNBH_3$) were added and dissolved into the solution. The lyophilized powder of HA oligosaccharide mixture was dissolved in double distilled water at a concentration of 1 mg/ml. 10μl of each oligosaccharide sample were incubated for 2 h in 65°C with 5 μl of

the above prepared 2AB solution and were then recovered by cooling and briefly centrifuging. 5 µl of this solution was applied to the bottom of a dry strip of Whatman 3MM chromatography paper, and was placed in a glass vessel containing acetonitrile for 1 h. The paper was dried and viewed under UV light. The spots on the chromatography paper were cut out and inserted into 3 ml syringes that were fitted with 0.45 µm Millex-LCR filters. The labeled oligosaccharides were extracted from these cut-outs by 4 elutions of 0.5 ml of double distilled water and were dried. Solutions of concentration of 1% of the samples were prepared (1 µl of sample, 19 µl of double distilled water and 80 µl of acetonitrile). A calibration ladder of a 2AB-labeled dextran hydrolysate was used to convert the elution times of the HA oligosaccharides into glucose units (GU), as described previously (27) The samples were injected to an Amide 80 column in High Pressure Liquid Chromatography (HPLC) for the determination of the size of the oligosaccharides.

Synthesis of Lys-HA$_{34}$

HA was converted to hyaluronic-acid tetrabutylammonium-bisulfate (HA-TBA), as described previously (28,29), and coupled to Lysine-(fmoc)-OH by reductive amination using $NaCNBH_3$ in dry DMSO. Briefly, HA-TBA, Lysine-(fmoc)-OH and $NaCNBH_3$ were mixed together at molar ratios of 1:2:40, respectively, and dissolved in DMSO to give a solution of concentration of 30 mM. The reaction was stirred for 5 days at 50°C, the product was precipitated in cold diethyl ether: acetone (1:2) and isolated. Subsequently, the dry precipitant was dissolved in double distilled water and Lys-(fmoc)-HA separated on Sephadex G-25 (PD-10) column (cut-off: 5 kDa). The end product was characterized by TLC R_f 0.9 ($CHCl_3$:MeOH:AcOH 5:3:1) and by ^{1}H-NMR 500 Hz (in D_2O) using the Fmoc protons chemical shift (δ 6.8–8.1, m, 8H) for the calculation. The assignments and chemical shifts of the ^{1}H signals of HA (D_2O) are in accordance with published results (30). Fmoc protecting group was removed by dissolving Lys-(fmoc)-HA in piperidine: DMF (1:3) solution.

Synthesis of PTX-LEV

PTX was esterified on C2′ of PTX with 4-oxopentanoic acid (levulinic acid, LEV) to afford the respective ester derivate, designated as PTX-LEV (Fig. 4), based on a previously described procedure (31). Briefly, DCC (0.182 mmol) and levulic acid (0.166 mmol) were dissolved in 300 µl DMF and cooled for 20 min to -18°C. Then a solution of PTX (0.117 mmol) and DMAP (0.117 mmol) was added dropwisely. The reaction was stirred at 4°C for 20 h. DCU precipitants were discarded and the clear solution was precipitated in cold double distilled water. PTX-LEV was characterized by MALDI-TOF. Found: 974.1 and 990.0, calculated for M + Na^+, and M + K^+, respectively.

Synthesis of Monomers

The monomers methacryloyl-glycylglycine (MA-GG-OH) (32) methacryloyl-glycylglycine p-nitrophenyl ester (MA-GG-ONp) (33), methacryloyl-glycylglycine hydrazide-Boc (MA-GG-HZBoc) (34), methacryloyl-aminopropyl-fluorescein-5-isothiocyanate (MA-AP–FITC) (35), and HPMA (36) were synthesised as described previously.

Synthesis of an Amine-Containing HPMA Copolymer Precursor (P-(AP)-FITC)

HPMA copolymer precursor having free amine groups for HA oligomers attachment (designated as P-(AP)-FITC, where P represents the HPMA copolymer backbone) was prepared by radical precipitation copolymerization of HPMA, MA-AP-FITC and N-(tert-butyloxycarbonyl-aminopropyl)methacrylamide (MA-AP(Boc)) in acetone/DMSO in a molar ratio of 89:1:10, respectively. The ratio of monomers to initiator and solvent was 12.5:0.6:86.9 wt%, respectively. The vial was sealed and placed in a water bath for 24 h at 55°C in the dark. The precipitated polymer was dissolved in methanol and precipitated into diethyl ether: acetone (1:2) isolated and desiccated. The dried polymer was dissolved in double distilled water, dialyzed using a dialysis bag (MWCO; 6000–8000 Da), and lyophilised. FITC loading was determined from its UV absorbance at 492 nm. The content of the Boc protected amine was determined by ^{1}H-NMR 500 Hz in D_2O, using the Boc *t*-butyl proton chemical shift (δ 1.40, s, 9H) for the calculation. The weight average molecular weight M_w, the number average molecular weight M_n and polydispersity (I) of the copolymer were determined by SEC in FPLC using HiPrep™ Sephacryl 16/60 S-400 column calibrated with HPMA standards. The characteristics of the polymers and the methods used for characterization are summarized in Table I. The Boc protecting groups were then removed by concentrated TFA.

Synthesis of FITC-Labeled HA-Modified HPMA Copolymers (P-(HA$_{4-14}$)-FITC)

Each HA oligosaccharide was conjugated to the P-(AP)-FITC copolymer precursor by reductive amination. P-(AP)-FITC, HA oligosaccharides and $NaCNBH_3$ were mixed together at molar ratios of 1: 5: 40, respectively, and dissolved in DMSO to give a solution of concentration of 20 mM. Each solution was incubated for a 72–96 h at 40°C in the dark. The solutions were then precipitated into diethyl ether: acetone (1:2), centrifuged and desiccated. Each dried

Table I Quantities of HA Oligomers Received and Their Corresponding Incubation Times

Incubation times (h)	Initial quantity of HA before degradation (mg)	HA_4 (mg)	HA_6 (mg)	HA_8 (mg)	HA_{10} (mg)	HA_{12} (mg)	HA_{14} (mg)
24	10.0	3.0	2.9	–	–	–	–
12	3.8	1.8	1.2	0.4	–	–	–
4	5.6	2.3	2.0	1.1	–	–	–
2	31.4	1.1	3.5	3.3	4.4	4.0	4.6
1	11.2	–	0.9	1.1	1.3	1.1	1.9
0.5	12.6	–	0.4	0.7	1.0	1.6	1.2

The table summarizes the quantities acquired for each fragment with respect to the initial quantity of HMW-HA that was used for the degradation. It is observable that each incubation time provides a different distribution of fragments with different quantities

conjugated polymer was dissolved in double distilled water and dialyzed against double distilled water (MW cutoff 6000–8000 Da) and lyophilized. The HA conjugation was confirmed by ^{1}H-NMR 500 Hz in D_2O using the HA chemical shift (δ 3.5–4.5). The uronic acid content of HA was determined according to a published protocol of the carbazole method (37). The characteristics of the polymers and the methods used for characterization are summarized in Table II.

Synthesis of p-Nitrophenyl- and Hydrazide-Containing Copolymer Precursor (P-(GG-ONp)-HZ-Boc)

The HPMA copolymer precursor with active ester groups and protected hydrazide groups (P-(GG-ONp)-HZBoc), which was later used for HA and PTX attachment, respectively, were synthesised, isolated, and purified as described previously (38). The mol% ratio of HPMA, MA-GG-ONp, and MA-GG-HZ-Boc was 80:10:10, respectively. The ratio of monomers to initiator and solvent was 12.5:0.6:86.9 wt%, respectively. The p-nitrophenol content of the polymers was determined after dissolving a known mass of the polymer in 1 ml 1 N NaOH for 1 h and reading absorbance at 400 nm in a TECAN Infinite M200 Plate Reader. An extinction coefficient of ε=17700 M^{-1} cm^{-1} was used for calculation. The number of HZ-Boc group content was determined by ^{1}H-NMR in D_2O according to Boc *t*-butyl proton chemical shift (δ 1.40, s, 9H). The characteristics of the polymers are summarised in Table III.

Synthesis of PTX-Containing HPMA Copolymers

P-(HA_{34})-PTX conjugates bearing HA oligosaccharide at the size of 34 monosaccharides was prepared in a three-steps procedure based on a previously described method. (38) Lys-HA_{34} was first attached to the polymeric backbone

Table II Characteristics of HA-Containing Copolymer Conjugates, P-(HA_{4-14})-FITC

Polymer conjugate	MW of HA fragment (Da)[a]	HA content in conjugate (wt%)[b]	HA content in conjugate (mol%)[c]	Free NH_2 content (mol%)[d]
P-(AP)-FITC [e, f, g] Precursor	–	–	–	9.9
P-(HA_4)-FITC	758	10.0	4.0	5.9
P-(HA_6)-FITC	1137	7.3	1.9	8.0
P-(HA_8)-FITC	1516	14.1	2.8	7.1
P-(HA_{10})-FITC	1895	11.6	1.8	8.1
P-(HA_{12})-FITC	2274	9.1	1.2	8.7
P-(HA_{14})-FITC	2653	8.1	0.9	9.0

[a] Calculated from the molecular weight of each saccharide in the oligosaccharide

[b] Determined by the carbazole method for the determination of uronic acid content

[c] Calculated from the wt% of the HA content

[d] Calculated by ninhydrin assay

[e] P designates the HPMA copolymer backbone

[f] AP designates the deprotected amine moiety in the polymer

[g] P-(AP)-FITC: Mw(51.6 kDa); Mn (31.6 kDa); I (1.63), FITC content (0.4 mol%) AP content (9.89 mol%)

Table III Characteristics of P-(HA_{34})-PTX and the Precursor Copolymer

Polymer code	Type of polymer	MW (kDa)[a]	I[a]	% mol ONp[b]	% mol HA_{34}[c]	% mol HZ-Boc[d]	% mol drug[e]
P-(GG-ONp)-HZ-Boc	precursor	12	1.05	2	–	9.95	
P-(HA_{34})-PTX	targeted	15	1.05	–	1.5		0.8
P-(GG-OH)-PTX	control	12	1.05	–			1.5

[a] Weight average molecular weights of copolymers were estimated by size-exclusion chromatography

[b] The content of ONp residues was determined spectrophotometrically

[c] The content of HA was estimated by Carbazol assay

[d] The content of Boc was estimated by ^{1}H-NMR at 500 Hz

[e] The content of PTX-LEV was determined by HPLC following an acidic cleavage

of P-(GG-ONp)-HZBoc) by aminolysis to give P-(HA_{34})-HZBoc. The Boc protecting groups were then removed by concentrated TFA. For the last step, the intermediate copolymer with free hydrazide groups, P-(HA_{34})-HZ was dissolved in anhydrous methanol to a 10 wt% solution, and 60 mol% PTX-LEV (relative to the hydrazide content) was added under stirring. The reaction was carried out for 48 h after adding catalytic amount of AcOH, and terminated by the precipitation of the polymer in diethyl ether. The control non-targeting PTX conjugate, P-PTX (without HA) was prepared by attaching PTX-LEV to P-(GG-ONp)-HZBoc precursor, from which the ONp groups were first released in 1 N sodium hydroxide solution. The conjugates were isolated and purified on LH-20 column using methanol as eluent. P-(HA_{34})-PTX and P-PTX conjugates were characterized by SEC on FPLC system, using Sephacryl S-400 column. The total content PTX derivative in polymer conjugates was determined by HPLC system (waters 2695), on a column RP-C18 (waters 4.6×250 mm), after complete hydrolysis of the polymer conjugates in HCl solution (pH ~ 2) for 1 h at 37°C and extraction of the derivatives to chloroform, as described (31).

Immunofluorescence Analysis for CD44 Expression

8×10^4 cells/ml of either SK-OV-3 or DU-145 were seeded in a 24-well plate already containing cover slips. After an incubation of 48 h at 37°C, the medium was aspirated, the cells were washed with PBS, fixated for 8 min with 4% paraformaldehyde (PFA), and blocked with 3% BSA for 30 min. The cells were then exposed to Alexa Fluor 647 conjugated anti-human CD44 antibody, (clone IM7) that was diluted 1:200 in 1% BSA and incubated at room temperature for 1 h (except the control cells that were treated with 1% BSA). Afterwards, cells were washed carefully and mounted in Mowiol-DABCO mounting medium containing DAPI. Images were acquired with an Olympus FV1000-IX81 confocal microscope (excitation at 650 nm, emission collected with a 668 nm barrier filter).

Copolymers Binding to Human Cell Lines

The fluorescence of FITC was used to monitor the binding of the HA conjugated copolymers to the cells. 2×10^4 cells/ml were seeded in a 24-well plate for 24–48 h. The medium was aspirated, a fresh polymer solution (200 mg/ml) was added to the cells and cells were incubated overnight at 37°C. The cells were trypsinized and the cell-associated fluorescence was determined immediately using flow cytometry (Guava MiniEasyCyte, λ_{ex}=485 nm and λ_{em}=525 nm). Gates for fluorescence measurements were set according to the control sample of untreated cells. The analysis of the results was performed with Cytosoft software (GUAVA Tech).

Subcellular Fate of the Targeted HPMA Copolymers in Human Cell Lines

Confocal microscopy was used to evaluate the intracellular fate of the FITC-labeled copolymer conjugates by CD44-expressing SK-OV-3. 8×10^4 cells were seeded in 24-well plates containing cover slips and incubated of 48 h in 37°C. The medium was then replaced with medium containing the copolymers (200 μg/ml) and the cells were incubated overnight. The medium was then aspirated, the cells were washed twice with PBS, fixated for 8 min with 4% PFA, and blocked with 3% BSA for 30 min. The cells were then exposed to Alexa Fluor 647 conjugated anti-human CD44 antibody (clone IM7) that was diluted 1:200 in 1% BSA and incubated at room temperature for 1 h (except the control cells that were treated with 1% BSA). Afterwards, cells were washed carefully and mounted in Mowiol-DABCO mounting medium containing DAPI. Images were obtained by Olympus FV1000-IX81 confocal microscope.

Cytotoxicity Assay

The cytotoxicity of free PTX, non-targetable P-PTX copolymer, and the targetable PTX conjugate P-

(HA_{34})-PTX against SK-OV-3, and DU-145 cells was assessed using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinium bromide (MTT) assay (39). All concentrations of polymer-drug conjugates are expressed in PTX equivalents. Briefly, cells were seeded into 96-well microtitre plates at a density of 5000 cells per well. Forty-eight hours after seeding, the medium was removed and 12 different concentrations of sterile products (free PTX, P-PTX and P-(HA_{34})-PTX) in fresh media were added for 6 h at 37°C. The drug solution was then removed and replaced with fresh medium, followed by 72 h of incubation at 37°C. Cell survival was assayed by discarding the medium and then adding 100 µl of fresh medium and 25 µl of 5 mg/ml MTT solution in PBS to each well and incubating for 4 h. The medium was discarded and 100 µl of DMSO were added to dissolve formazan crystals. The absorbance of each sample was measured at 570 nm. The results of the cytotoxicity assay were used to calculate the IC_{50} relative to a control of non-treated cells. The cytotoxicity is expressed as the equivalents of PTX that kills 50% of cells (IC_{50}).

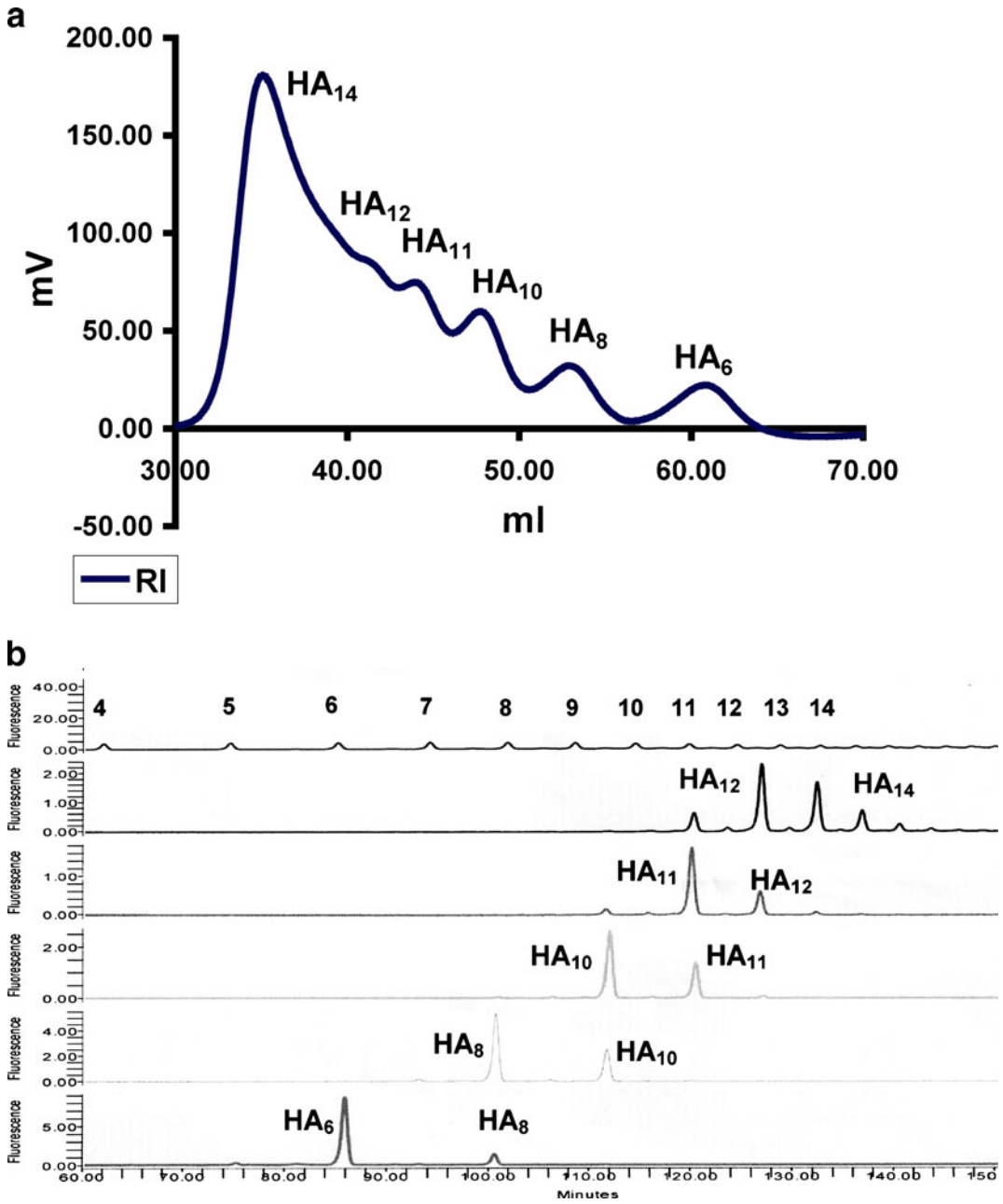


Fig. 1 Characterization of HA oligomers obtained by enzymatic digestion of HMW-HA. (**a**) An example for size exclusion chromatogram received for an incubation of 1 h in 37°C. (**b**) HPLC analysis performed for each fraction.

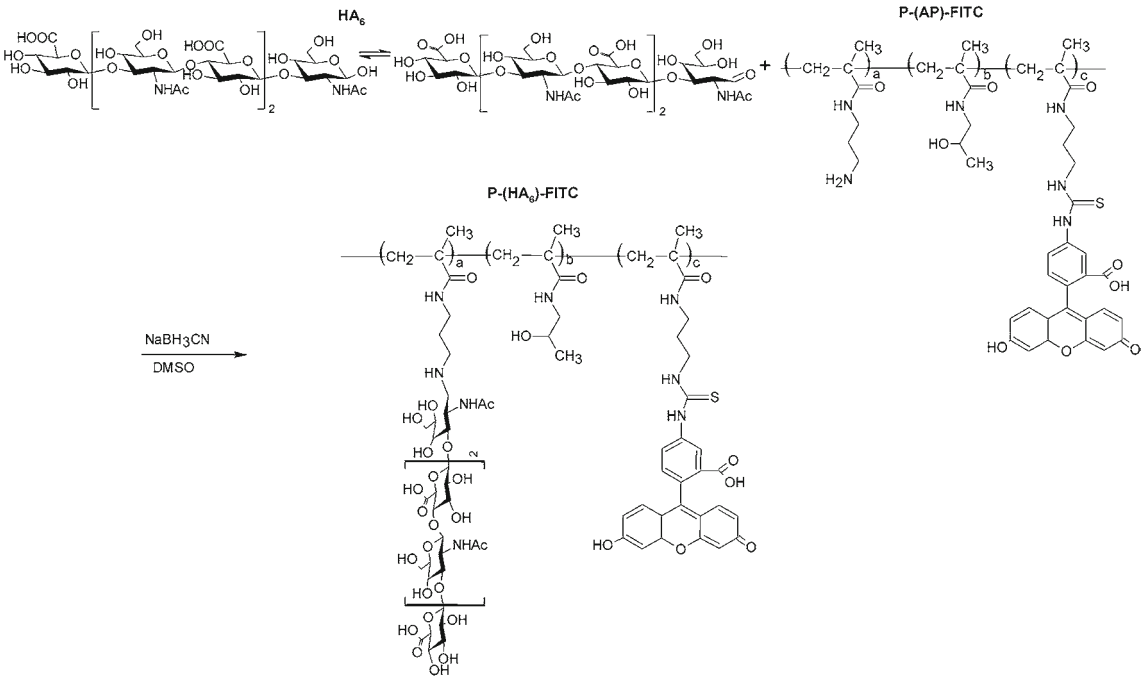


Fig. 2 Synthesis of the FITC-labeled HPMA copolymers conjugated with LMW-HA oligomers, P-(HA_{4-14})-FITC. Here shown is an example of the conjugation of the hexasaccharide HA_6 to the copolymeric precursor P-(AP)-FITC by reductive amination.

RESULTS AND DISCUSSION

HA-drug conjugate have been previously designed for the targeted delivery of PTX to cancer cells (40). PTX was conjugated to the HA via an adipic dihydrazide (ADH) linker, whose content on the HA was corresponding to different percentages of the PTX in the conjugate. The HA-PTX conjugates showed selective toxicity toward the human cancer cell lines (breast, colon, and ovarian) that are known to over express CD44, while no toxicity was observed toward a mouse fibroblast cell line at the same concentrations used with the cancer cells. However HA-PTX conjugate with the lowest drug loading was more toxic than HA-PTX conjugates with higher drug loading. It was concluded that the decreased solubility of the conjugate with high PTX loading masked the recognition elements of HA, and thus limited the toxicity of the conjugate.

In this work we aimed at creating a water-soluble HA-modified delivery system that would demonstrate strong binding affinity to the CD44 receptors expressed on the ovarian cancer cells. To this end HMW-HA was enzymatically degraded by hyaluronate lyase at different incubation times to acquire a mixture of LMW-HA oligosaccharides. The mixture was separated using a size exclusion column in FPLC, in which chromatograms of the different sizes of HA oligosaccharides were received according to the different incubation times (Table I). Six HA oligomers of MW smaller than 3 kDa were attained. The sizes were

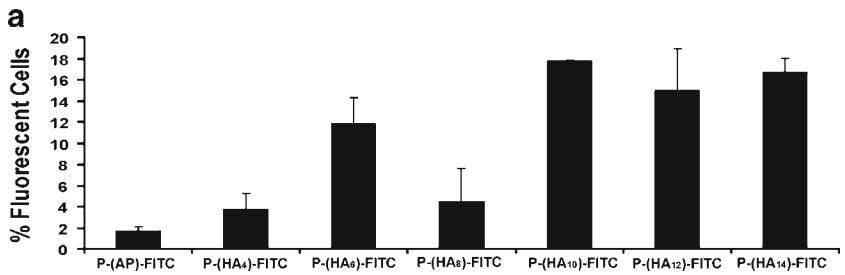
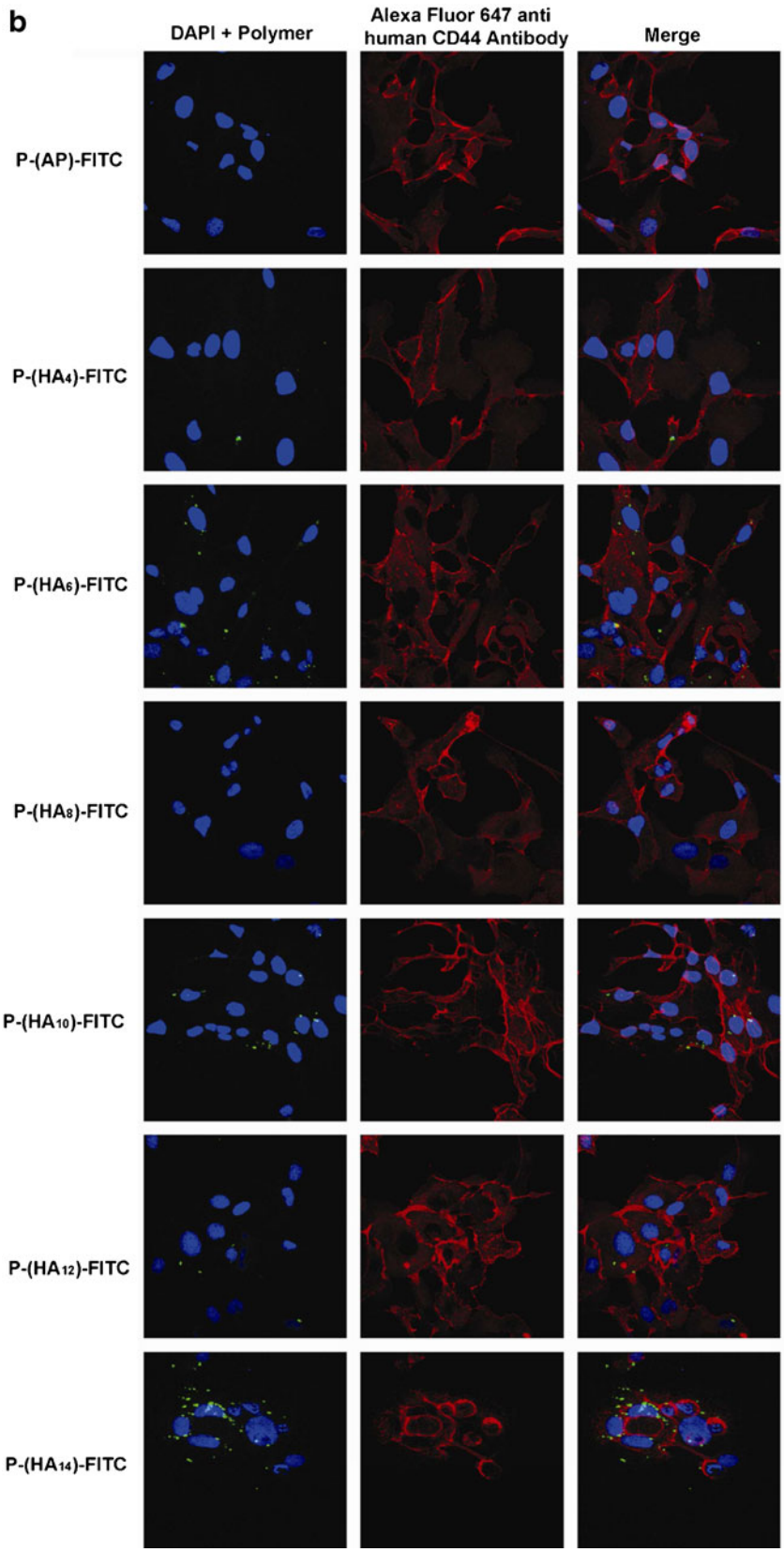


Fig. 3 (**a**) Flow cytometry assessment of the binding capability of P-(HA_{4-14})-FITC to CD44-over-expressing ovarian cancer cells. SK-OV-3 were incubated with P-(HA_{4-14})-FITC for 15 h at 37°C. The results are presented as percentages of fluorescent cells with respect to non-treated cells. The results shown are mean ± SD. (**b**) Representative confocal fluorescence images of SK-OV-3 cells incubated overnight at 37°C with the P-(HA_{4-14})-FITC conjugates and stained with Alexa Fluor 647 conjugated to anti-human CD44 antibody. The images show a merge of DAPI (blue, nuclear staining) and FITC (green, HA-containing conjugates internalized into the cells)—*DAPI + polymer*; Membrane CD44 specific staining (red, Alexa Fluor 647)—*Alexa Fluor 647 anti-human CD44* antibody; and merge of all the three colors - *Merge*.

Fig. 3 (continued).



determined by the HPLC analysis with dextran as a reference. An example of the FPLC and HPLC analysis for size determination of HA oligomers of each peak detected in the chromatogram is also shown (Fig. 1). The LMW-HA oligomers were then conjugated to FITC-labeled HPMA copolymer precursor bearing a free amine groups, via reductive amination (Fig. 2).

All conjugates bearing the HA_4-HA_{14} oligomers were evaluated for their binding ability and intracellular fate in CD44-overexpressing SK-OV-3 cells by means of flow cytometry and confocal fluorescence imaging (Fig. 3). The results confirmed that polymer conjugates bearing oligomers at the size of HA_{10} oligomer and above bind significantly (10-time higher than the non-targeted control copolymer) to the CD44 receptor (Fig. 3a). This is in agreement with previous literature reports, indicating HA_{10} to be the smallest oligosaccharide that binds significantly to the cell surface CD44, suggesting that HA binding site of CD44 is not optimally occupied until the oligomer size reaches HA_{10} (41).

Confocal images revealed that the extent of internalization of the HA_{14} copolymers by CD44-overexpressing cells was significantly higher than observed for conjugates bearing smaller $HA_{4\text{-}12}$ oligomers (Fig. 3), thus providing evidence for the CD44-mediated internalization of HA-modified copolymers into the ovarian cancer cells.

Comparing the composition of each copolymer conjugate reveals that there is also a certain quantity of free amine (aminopropyl-) that was not occupied by the HA oligosaccharides (Table II). This amine, due to its positive charge, can possibly bind, non-specifically, to the negatively-charged cell membrane and be taken-up by SK-OV-3 cells, and therefore affect the binding results. Had this been solely the case, without any contribution of the different HA oligomers to their uptake to the cells, there should have been an increase in fluorescence which corresponds to the increase in amine content in each conjugate. The control measurements showed low binding of the non-targeted copolymers (P-(AP)-FITC), with the highest free amine content, to the cells (% of labeled cells was less than 2). Furthermore, the results show a clear rise in fluorescence for the conjugate bearing the $HA_{10\text{-}14}$ oligomers compared to the other conjugates, and does not show a significant increase in the contents of the free amine. We assume that the excess of carboxyl groups of HA are capable of masking the positive charge of the un-occupied amine groups, and thus a direct correlation between the HA oligomer length and the

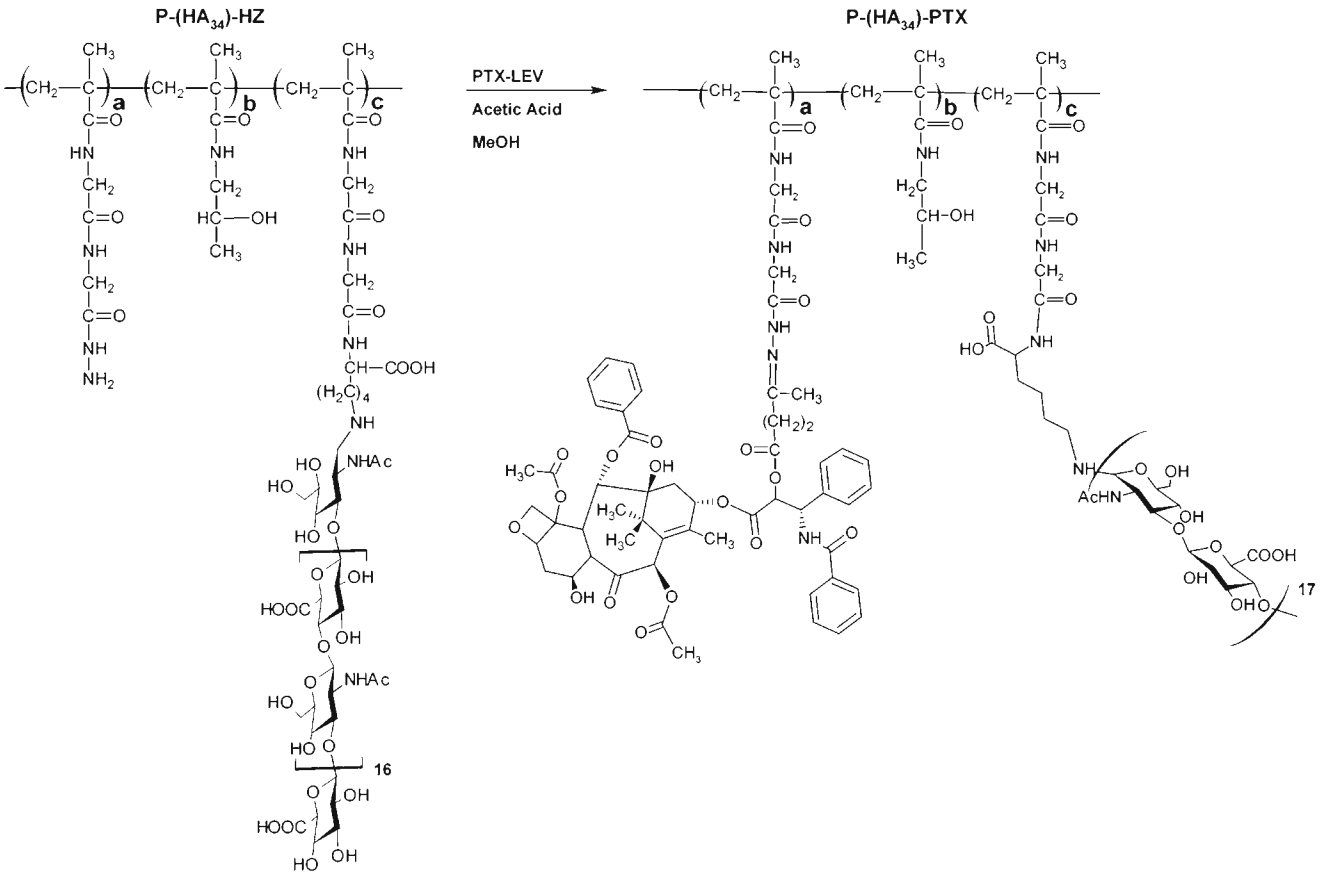


Fig. 4 Synthesis of the HA_{34} containing copolymer bearing PTX via a pH-sensitive hydrazone bond, P-(HA_{34})-PTX.

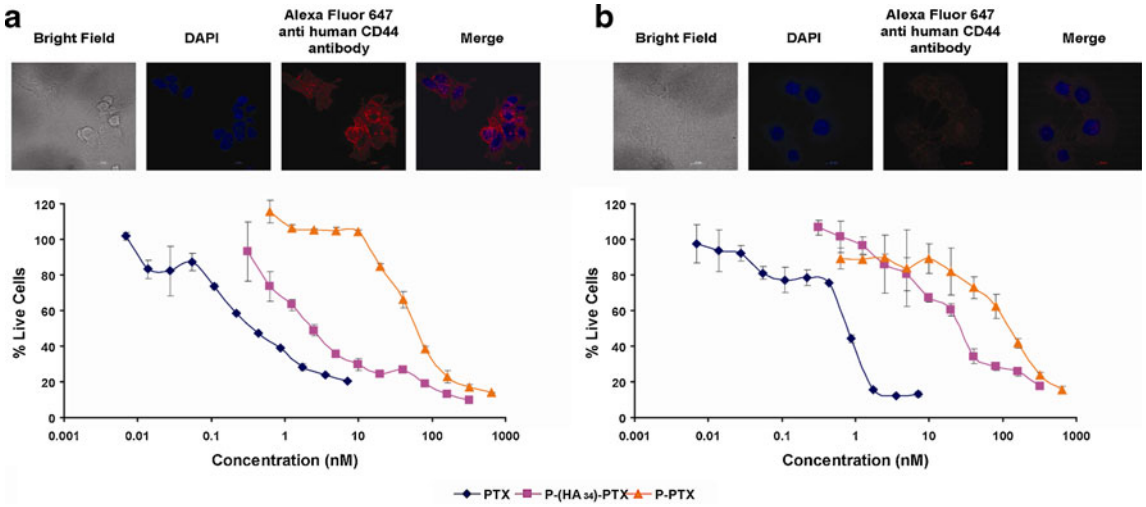


Fig. 5 *In vitro* cytotoxicity of free PTX (*black diamond suit*), P-HA_{34}-PTX (*black square*), P-PTX (*black up-pointing triangle*) conjugates against cells with high (SK-OV-3) (**a**) and low levels (DU-145) (**b**) of CD44. Cell viability of SK-OV-3 and DU-145 cells as function of PTX equivalent concentration. The cytotoxicity of the free drug and the polymer conjugates (targeted and non-targeted) were determined using the MTT assay.

binding and internalization into the SK-OV-3 cells was observed.

The improved binding and internalization of the HA_{10}-HA_{14} containing conjugate could be explained by interactions of the oligosaccharide with the binding domain of the CD44 receptor. Teriete *et al.* have previously explained that the CD44 receptor shows a substantial preference for HA due to the molecular recognition of the N-acetyl group of GlcNAc and carboxylate group of GlcUA at the sixth and seventh saccharide units in the HA chain, respectively (42). The hydrogen bonds formed at a location corresponding to these saccharides with the amino acids in the binding domain create a high affinity for binding and can explain why HA_{10}, which has two possible locations within its chain, shows a greater binding ability to the receptor than the smaller HA oligomers. The above explanation does not apply to HA_4 and HA_8, which did not show a high binding affinity to the receptor, since they are not long enough for all the interactions of the saccharides with the binding domain to apply. It is possible that the HA_{10}-HA_{14} oligomers represent divalent binding of the saccharide chains by two adjacent binding sites, an effect that is not observed by smaller HA oligomers. Since the polymer conjugates containing the $HA_{10\text{-}14}$ oligomers bind most effectively to the receptor, these copolymers are internalized to a greater extent than HA-containing conjugates with HA oligomer of minimum length of 4–8 saccharides.

Since an improved binding and intracellular uptake into CD44-overexpressing SK-OV-3 was demonstrated with increasing the size of the HA oligomers from 10 oligosaccharide units and above (Fig. 3), subsequent cytotoxicity experiments were performed with PTX-copolymer conjugate bearing HA_{34}, designated as P-(HA_{34})-PTX (Fig. 4). The mass average molecular mass (Mw) of P-(HA_{34})-PTX and the molecular mass distribution (I) were 15,000 and 1.05, respectively. The relatively low Mw of the precursor copolymer P-(GG-ONp)-HZ-Boc (12,000) is attributable to the high molar percentage of MA-GG-ONp in the monomer mixture (10 mol%) that served as a chain transfer agent (43). The cytotoxicity of P-(HA_{34})-PTX was studied by following its growth inhibitory activity against cells with high (SK-OV-3), and low (DU-145) levels of and CD44 expression, after 6 h exposure to the copolymer and an additional 72 h of incubation and comparing it to that obtained for the same experiments with free PTX, and with P-PTX. The lowest IC_{50} doses were calculated for the activity of free PTX in those two cell lines owing to the unimpeded diffusion of free PTX into the cells (Fig. 5, Table IV). This is in agreement with previous literature data (44). The CD44-targeted conjugate (P-(HA_{34})-PTX) was 50 times more toxic towards cells expressing high level of CD44 (SK-OV-3) relative to the non-targeted P-PTX, indicating specific delivery of the cytotoxic copolymer into the cells. P-(HA_{34})-PTX was only 6 times more toxic towards DU-145 cells

Table IV IC_{50} Values (in nM) of Free PTX and PTX-Containing HPMA Copolymers in CD44 (+) SKOV-3, and CD44(−) DU-145 Cells

Polymer code	Type of polymer	SK-OV-3	DU-145
PTX	Free drug	0.44 ± 0.1	0.88 ± 0.25
P-(HA_{34})-PTX	Targeted	1.2 ± 0.35	9.4 ± 0.65
P-PTX	Non-targeted	60 ± 0.1	60 ± 0.1

IC_{50} values were evaluated as the concentration of PTX that inhibits cell growth to 50% of the control cell level. The results shown are the mean values of the IC_{50} doses ± SD of three experiments, in duplicates

with low level of CD44 relative to the non-targeted P-PTX. Since the HA oligomers binding specificity to CD44-expressing cells was confirmed (Figs. 3 and 4), we assume that the higher toxicity of P-(HA_{34})-PTX towards SK-OV-3 cells may be attributed to the CD44-mediated endocytosis of the targeted conjugate and improve cytotoxicity.

CONCLUSIONS

LMW-HA oligosaccharides of sizes 4–14 sugar residues were successfully obtained by enzymatic degradation of HMW-HA by hyaluronate lyase and further attached to a fluorescently-labeled HPMA copolymer conjugates. Polymer conjugates bearing HA oligomers at the size of 10 monosaccharides and above, bind most profoundly to CD44-overexpressing cells and internalize to the greatest extent compared to the smaller size HA-containing conjugates, and thus can be used as a potential drug carrier for CD44- overexpressing ovarian carcinoma cells. P-$(HA)_{34}$-PTX conjugate exhibited 50-times higher cytotoxicity towards CD44-overexpressing cells relative to the control, non-targeted, P-PTX, and was less toxic towards cells with low level of CD44. The cytotoxicity of P-(HA_{34})-PTX requires cellular uptake of the copolymer followed by the release of the active PTX moieties in endosomal and lysosomal compartments. We therefore conclude that the HA_{34} is capable of delivering more PTX to CD44-overexpressing cells than to those cells with little or no CD44 expression.

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