

HPMA Copolymer-Based Polymer Conjugates for the Delivery and Controlled Release of Retinoids

O. LIDICKÝ¹, M. ŠÍROVÁ², T. ETRYCH¹

¹Institute of Macromolecular Chemistry of the Czech Academy of Sciences, Prague, Czech Republic, ²Institute of Microbiology of the Czech Academy of Sciences, Prague, Czech Republic

Received July 14, 2016

Accepted July 14, 2016

Summary

In this paper, we describe the synthesis, physicochemical characterization, drug release kinetics and preliminary biological evaluation of several *N*-(2-hydroxypropyl)methacrylamide (HPMA)-based polymer-retinoid conjugates designed for solid tumor immunotherapy. The conjugates are supposed to inhibit the immunosuppressive activity of myeloid-derived suppressor cells (MDSC) accumulated in the solid tumor microenvironment. All-trans retinoic acid (ATRA) was derivatized to hydrazide (AtrHy) and then attached to the polymer backbone *via* a spacer that is stable at the normal pH of blood (7.4) and hydrolytically degradable in mildly acidic environments (e.g. in endosomes or lysosomes, pH~5.0-6.5). Polymer-AtrHy conjugates were designed to achieve prolonged blood circulation and release of the immunomodulator intracellularly or extracellularly in solid tumor tissue. Three types of polymer precursors, differing in the structure of the keto acid-containing side chains, were synthesized. A linkage susceptible to hydrolytic cleavage was formed by the conjugation reaction of the carbonyl group-terminated side chains of the polymer precursors with the hydrazide group of a drug derivative. *In vitro* incubation of the conjugates in buffers resulted in much faster release of the drugs or their derivatives from the polymer at pH 5.0 than at pH 7.4, with the rate depending on the detailed structure of the spacer. Both the AtrHy derivative and its polymer conjugates showed the ability to induce the differentiation of retinoid-responsive HL-60 cells, thus demonstrating the required biological activity.

Key words

Polymer conjugate • Retinoid • HPMA • Controlled release • Myeloid-derived suppressor cells

Corresponding author

T. Etrych, Institute of Macromolecular Chemistry of the Czech Academy of Sciences, Heyrovského nám. 2, 162 06 Prague 6, Czech Republic. E-mail: etrych@imc.cas.cz

Introduction

In the last 30 years, many studies have focused on drug delivery to solid tumors using water-soluble *N*-(2-hydroxypropyl)methacrylamide (HPMA)-based polymer carriers. The advantages of such systems include the increased solubility of drugs after attachment to the polymer backbone, specific tumor targeting, reduction of drug side effects and significant prolongation of the blood circulation (Kopeček and Kopečková 2010, Ulbrich and Šubr 2010). Thanks to the increased molecular weight, the delivery system is passively targeted and accumulated in the tumor tissue due to the enhanced permeability and retention effect (EPR) (Fang *et al.* 2011, Seymour *et al.* 1995). Drugs can be covalently attached to the polymer carrier *via* various stimulus-sensitive spacers, e.g. spacers containing pH-sensitive hydrazone bonds, enzymatically degradable oligopeptides or reductively degradable disulfide bonds, which are designed to control the release of the drug from the carrier (Wicki *et al.* 2015).

Many anti-cancer drugs have been developed, but their therapeutic use still remains rather ineffective. One of the reasons is the frequent presence of various mechanisms that suppress anti-cancer immune responses, impairing the T-cell-dependent cytotoxic immune responses and allowing the tumor to escape the immune-mediated control. The current trend in cancer therapy is to counteract the suppressive mechanisms and to restore

the anti-cancer immune response (Kapadia *et al.* 2015) using synergistic combinations of anti-cancer treatment and immunomodulation. This strategy enables the generation of a specific anti-cancer immune response and the induction of persistent immunological memory (Vanneman and Dranoff 2012).

One of the suppressive mechanisms contributing to tumor-induced immune escape is mediated by a heterologous population of myeloid-derived suppressor cells (MDSC). They comprise immature granulocytes, macrophages, and dendritic cells and are endowed with a robust suppressive capacity (Gabrilovich and Nagaraj 2009, Ostrand-Rosenberg *et al.* 2009). MDSCs migrate from bone marrow through the blood and accumulate in secondary lymphoid organs and tumors. In normal healthy individuals, the immature myeloid cells differentiate to their mature functional counterparts. In the tumor microenvironment, their normal differentiation is prevented, and the cells acquire a potent suppressive activity towards both innate and adaptive immune responses *via* multiple mechanisms. These MDSCs are responsible for the suppression of T cells and natural killer (NK) cells. MDSCs sustain tumor progression by providing a favorable microenvironment in which transformed tumor cells can proliferate, acquire new mutations, and evade host immunosurveillance (Serafini 2013). MDSCs also contribute to tumor neoangiogenesis (Dolcetti *et al.* 2008). Increase in the systemic circulation of MDSCs is often associated with poor patient prognosis in various cancers (Weide *et al.* 2014). Therefore, MDSCs have attracted considerable interest as potential targets in the development of cancer therapies that may abrogate tumor-associated immunosuppression (Ugel *et al.* 2009). MDSC targeting in experimental murine tumors using anti-Gr-1 antibody demonstrated significant improvement in immune response, providing a proof of concept that reducing MDSC accumulation or suppressive function may potentiate anti-tumor therapy (Srivastava *et al.* 2012).

One of the drugs that could be used to reduce the amount of MDSCs is all-trans retinoic acid (ATRA). In tumor-bearing mice, the CD4⁺- and CD8⁺-mediated tumor-specific immune responses were noticeably improved after ATRA treatment. ATRA induces the differentiation of MDSCs into mature myeloid cells (macrophages, dendritic cells, or granulocytes) by the up-regulation of glutathione synthesis and down-regulation of reactive oxygen species (ROS) (Kusmartsev *et al.* 2003, Nefedova *et al.* 2007). The treatment of mice with

ATRA decreased the spleen MDSCs, reduced the blood MDSCs in patients with renal carcinoma, and reversed the immune suppression (Mirza *et al.* 2006, Kusmartsev *et al.* 2008). ATRA is a hydrophobic compound that is UV and oxygen sensitive. Moreover, as a low-molecular-weight agent, ATRA has no selectivity towards tumor tissue. Thus, its applicability as a selective treatment agent capable of modulating MDSC activity in solid tumors is limited. Its stability, solubility, bioavailability and biodistribution could be significantly improved by using nanocarriers. Several methods have been published: the use of micelles (Fattahi *et al.* 2012) or microspheres encapsulated with ATRA (Jeong *et al.* 2003), a solid lipid nanoparticle powder formulation of ATRA (Lim *et al.* 2004), or block copolymer nanoparticles loaded with ATRA (Tiwari *et al.* 2011).

In this work, we have focused on the preparation of polymer systems based on the HPMA copolymers that are suitable to covalently bound ATRA, deliver it and release in controlled manner within solid tumor tissue. The polymer-based tumor-specific delivery of ATRA is designed for tumor-targeted immunomodulation that could potentially be explored to eliminate immune suppression and to potentiate anti-tumor therapy.

Materials and Methods

Chemicals

1-Aminopropan-2-ol, methacryloyl chloride, 2,2'-azobis(isobutyronitrile) (AIBN), 4,4'-Azobis(4-cyanovaleric acid) (ABIK), dimethylformamide (DMF), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysuccinimide, thiazolidine-2-thion (TT), orthophthalaldehyde (OPA), *N*-ethyl-diisopropylamine, dimethyl sulfoxide (DMSO), 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (ACVA-RAFT), 4-oxo-pentanoic acid (OPe) and *N,N'*-dicyclohexylcarbodiimide (DCC) were purchased from Fluka. 2,4,6-Trinitrobenzene-1-sulfonic acid (TNBSA) was purchased from Serva. *N*-(tert-butoxycarbonylamino)propylmethacrylamide (APMA-BOC) were purchased from Polysciences. All-trans retinoic acid (ATRA) was purchased from Enzo life science. 4-oxo-4-(2-pyridyl)butyric acid (OPB) and 5-cyclohexyl-5-oxovaleric acid (COV) were purchased from Rieke®Fine chemicals. RPMI 1640 medium, L-glutamine and antibiotics (penicillin/streptomycin) were purchased from Sigma-Aldrich. Fetal calf serum (FCS) was purchased from Invitrogen.

Synthesis of monomer

HPMA was synthesized as described in (Ulbrich *et al.* 2000); elemental analysis: calc./found C=58.72/58.98 %; H=9.15/9.18 %; N=9.78/9.82 %.

Synthesis of polymer precursor

Polymer precursor Pol was prepared by controlled RAFT radical polymerization carried out at 70 °C for 17 h using AIBN as an initiator and ACVA-RAFT as a RAFT chain transfer agent in mixture of dioxane:distilled water 1:2 [dioxane (3.88 ml) and distilled water (7.76 ml)] as the solvent. The molar ratio of HPMA and APMA-BOC monomers was 9:1; 10.49 mmol HPMA (1,500 mg) and 1.17 mmol APMA-BOC (270 mg). Afterward the polymer was obtained by precipitation in acetone:diethyl ether (2:1). Dithiobenzoate chain-terminating groups were removed by reaction of polymer precursor with AIBN in DMF at 80 °C for 3 hours. Polymer product was isolated by precipitation in acetone and reprecipitation in diethyl ether:acetone (1:2) mixture. Protecting BOC groups were removed by dissolving the polymer precursor overnight in methanol/HCl solution. Amount of amino groups in polymer precursor Pol-NH₂ was determined using UV/VIS spectroscopy *via* modified TNBSA assay as described previously (Etrych *et al.* 2001) using absorption coefficient $\epsilon_{420} = 11,550 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$.

Synthesis of polymer Pol-OPB

The synthesis of the polymer precursor containing side chains terminating in the OPB moiety was performed in two steps. Briefly, in the first step, the thioazolidin-2-thion (TT) amide of OPB was prepared using carbodiimide chemistry. OPB (31 mg, 0.173 mmol) was dissolved in 600 μl of DCM and mixed with a solution of EDC (33.3 mg, 0.214 mmol) and TT (21 mg, 0.176 mmol) in 1.7 ml of DCM. The reaction was monitored using an HPLC Shimadzu system equipped with a DAD detector. After 3 h, OPB-TT was purified by extraction with NaHCO₃ in H₂O (acetate buffer 5.5), filtered and dried with Na₂SO₄, evaporated and dissolved in methanol. The solution of OPB-TT in DMF (41 mg) was afterward immediately added to the polymer precursor Pol-NH₂ (160 mg) dissolved in 8.5 ml of DMF and left overnight at 8 °C. The polymer Pol-OPB was purified from low-molecular impurities by column chromatography using Sephadex LH-20 with methanol as the mobile phase. The product was obtained by precipitation into diethyl ether. The amount of OPB was

determined indirectly based on the amount of amino groups on the polymer precursor before and after the reaction with OPB-TT. Finally, the remaining amino groups in Pol-OPB were reacted with acetic anhydride to block them before the subsequent reactions. Yield: 158 mg (91 %).

Synthesis of polymer Pol-COV

The synthesis of the polymer precursor containing side chains terminating in COV was conducted in two steps, as described above. Briefly, in the first step, the TT containing reactive intermediate product of COV was prepared by the same method described above, using EDC as the carbodiimide coupling agent. The solution of COV-TT (76.8 mg in 1 ml of DMF) was afterward immediately added to the solution of polymer precursor Pol-NH₂ (300 mg in 6 ml of DMF) followed by the addition of DIPEA (27 μl) and left overnight at 8 °C. After the removal of the low-molecular-weight impurities by column chromatography and precipitation into diethylether, the amount of COV was determined indirectly as described for Pol-OPB. Finally, the remaining amino groups in Pol-COV were reacted with acetic anhydride to block them before the subsequent reactions. Yield: 264 mg (83 %).

Synthesis of polymer Pol-OPE

In the first step, the amino reactive 1-(2-thioxo-thiazolidin-3-yl)-pentane-1,4-dione (OPE-TT) was prepared as described previously (Šubr *et al.* 2014). Briefly, 4-oxo-pentanoic acid and TT were dissolved in dichloromethane (DCM), and EDC was added as a coupling agent under vigorous stirring. The reaction was stirred for 3 h at room temperature, and the product was extracted with distilled water and 2 wt.% NaHCO₃ in aqueous solution. The final product was obtained by crystallization from ethyl acetate. Elemental analysis: calc./found C=44.22/44.34 %; H=5.10/5.23 %; N=6.45/6.47 %; S=29.51/28.52 %; ¹H NMR 300 MHz (DMSO, 296 K). Polymer Pol-OPE was prepared by the reaction of polymer precursor Pol-NH₂ (200 mg) dissolved in 3 ml of DMF with OPE-TT (100 mg) dissolved in 1 ml of DMF, carried out in the presence of 15.2 μl of DIPEA. After 3 h, the polymer was purified from low-molecular-weight impurities by liquid chromatography using a column filled with Sephadex LH-20 with methanol as the eluent. The polymer Pol-OPE was precipitated into ethyl acetate. Yield: 190 mg (73 %).

Synthesis of hydrazide derivative of ATRA

All trans-retinoic acid (116 mg, 0.386 mol), DCC (87.6 mg, 0.425 mol) and *N*-hydroxysuccinimide (47 mg, 0.408 mol) were dissolved in mixture of DCM (1 ml) and DMF (0.1 ml) at RT in the dark under an argon atmosphere. After 96 hours, the precipitated dicyclohexylurea was filtrated out, and hydrazine hydrate (58 mg, 1.8 mmol) was added dropwise. The reaction was carried out using an HPLC system as described above. After one hour, impurities were removed by column chromatography on silica gel using a mixture of ethyl acetate:hexane (20:3) as the mobile phase. The second fraction containing the products was collected, and the ATRA hydrazide derivative (AtrHy) was obtained after evaporation of the solvent. Yield and characterization: 107 mg (88 %), elemental analysis: calc./found C=76.39/73.66 %; H=9.62/10.52 %; N=8.91/9.4 %; O=5.09/6.48 %; ¹H NMR 300 MHz (CDCl₃, 295 K).

Synthesis of polymer conjugates containing AtrHy

Polymer conjugates Pol-OPE-AtrHy, Pol-OPB-AtrHy and Pol-COV-AtrHy containing the AtrHy derivative were prepared by the reaction of the hydrazide group of the ATRA derivative with the respective polymer precursors containing keto groups. The reaction was carried out in methanol with the addition of acetic acid (40 µl/ml methanol) in the dark and under inert atmosphere (Etrych *et al.* 2002). The unreacted AtrHy derivative was removed from the polymer conjugates by the precipitation of the polymer conjugate into diethyl ether. Yield: 93 mg Pol-OPE-AtrHy containing 8.6 mg AtrHy (75 %), 87 mg Pol-OPB-AtrHy containing 7.2 mg AtrHy (56.8 %), and 35 mg Pol-COV-AtrHy containing 2.7 mg AtrHy (62.6 %).

Physico-chemical characterization

Content of ATRA or its derivate was determined by UV/Vis spectrophotometry on Helios α (Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer ($\epsilon_{363}=22,690 \text{ l}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ in methanol). Determination of molecular weight and dispersity of polymer precursors and polymer conjugates was carried out with an HPLC Shimadzu system equipped with RI, UV, and multi-angle light scattering DAWN EOS (Wyatt, USA) detectors using 0.3 M acetate buffer pH 6.5 and GPC column TSKgel G3000SWxl (300×7.8 mm; 5 µm); mobile phase containing 20 % of 0.3 M acetate buffer (CH₃COONa/CH₃COOH; pH 6.5; 0.5 g/l NaN₃) and 80 % of methanol; flow rate 0.5 ml/min). Hydrodynamic radius (R_h) of

polymer samples dissolved in phosphate buffer (0.1 M, with 0.05 M NaCl; polymer concentrations: 0.01 g/ml⁻¹) was measured at 25 °C by dynamic light scattering (DLS) using Nano-ZS instrument Zetasizer (ZEN3600, Malvern) as described previously (Chytil *et al.* 2010). The values were a mean of at least five independent measurements.

In vitro release study

The rate of AtrHy release from the polymer conjugates was measured in phosphate buffers modeling the environment in endosomes and lysosomes (pH 5.0) or in the blood stream (pH 7.4) at 37 °C using an HPLC Shimadzu system equipped with a DAD detector and reverse phase Chromolith®HR RP-18 endcapped 100-4.6 HPLC column. The appropriate polymer conjugate was dissolved in buffer at a final concentration equivalent to 5 µM AtrHy. At predetermined time intervals, 20 µl of the solution were analyzed by HPLC to determine the contents of free, released, and polymer-bound AtrHy.

Proliferation assay

Human promyelocytic leukemia cells from cell line HL-60 (ATCC CCL-240) were used as a prototype ATRA-responsive cell line. The cells were grown in RPMI 1640 medium (Sigma Aldrich) supplemented with 2 mM L-glutamine, antibiotics (pen./strept., Sigma Aldrich) and 10 % fetal calf serum (FCS; Invitrogen). The cells were plated onto 96-well plates (Thermo Scientific), 5×10³ cells/well, and cultivated with various concentrations of the conjugates, ATRA and AtrHy as control. The conjugates and free drugs were dissolved in DMSO at a concentration of 10 mM ATRA or AtrHy, and further dilution was performed using the culture medium. All sample handling was limited to minimal time and lighting. Next, the proliferation was determined using the standard ³H-thymidine incorporation assay (Říhová *et al.* 2001). The cells were pulsed with 30 kBq during the last 6 hours of the total three-day cultivation period. All measurements were performed in quadruplicate, and the results were calculated as the percentage of untreated control cells.

Results and Discussion

In this paper, we present the results of the synthesis, physicochemical characterization and evaluation of preliminary biological activity of polymer conjugates containing covalently bound ATRA

derivative, AtrHy, as a biologically active compound. A water-soluble polymer carrier was used in this study with the aim to increase the solubility of ATRA, prolong blood circulation within the body, and enable the passive accumulation and controlled release of the ATRA derivative within the solid tumor mass. The local increase of retinoid concentration in the solid tumor should downregulate the accumulation of MDCSs by stimulating their differentiation. Upon changing to a more differentiated status, MDSCs downregulate their immunosuppressive activity. Thus, the proper CD8⁺-mediated immune response could be restored. As a suitable polymer carrier, we used linear polymer precursors based on HPMA copolymers. The side chains contained amino groups modified by three different keto acids with varied structures. The difference in the chemical structure in the vicinity of the keto group affects the stability of the pH-sensitive hydrazone bond between the AtrHy derivative and the polymer carrier and could be used to regulate the AtrHy release kinetics, as our observations confirmed.

Synthesis of polymer conjugates

The polymer precursor Pol was prepared by the controlled RAFT copolymerization of HPMA and APMA-BOC monomers. The copolymer showed characteristics suitable for a polymer carrier in drug delivery, i.e. very narrow dispersity $\bar{D}=1.13$ and a molecular weight of 25,000 g/mol (Table 1). The removal of the BOC protecting groups did not significantly change the dispersity and molecular weight of the polymer precursor Pol-NH₂.

Generally, hydrazone bond is formed between hydrazide and keto groups in mild acidic conditions. To prepare polymer with a pH-sensitive hydrazone bond bound retinoid (AtrHy), the polymer precursor Pol-NH₂ and ATRA had to be modified before the final syntheses. The polymer Pol-NH₂ was modified with three different keto acids with varying structure, OPe, OPB and COV, with the aim of obtaining different release rate of AtrHy under conditions mimicking the body environment. Generally, each keto acid was first activated to its amino-reactive TT-derivate by the carbodiimide coupling method. Afterward, the reactive derivatives were used to modify the amino groups presented on the polymer precursor Pol-NH₂. The modification by keto acids did not significantly change the dispersity and molecular weight of the respective polymer precursors. The yield of the reaction varied among the keto acids, being the

highest for OPB (91 %) and slightly lower for OPe (73 %) and COV (71 %). The remaining amino groups in polymer precursors Pol-OPe, Pol-OPB and Pol-COV were protected by reaction with acetic anhydride to prevent crosslinking reactions of the amino groups and the keto groups on the polymers.

ATRA was first activated using carbodiimide chemistry and then reacted with an excess of hydrazine hydrate to avoid the bis-modification of hydrazine hydrate. Due to the UV and oxygen sensitivity of ATRA, the reaction was carried out in the dark and under inert conditions. The reaction course was monitored by an HPLC equipped with a DAD detector, and the reaction was stopped upon reaching a high yield.

The final polymer conjugates were prepared by the reaction of AtrHy and the respective polymer precursor containing keto groups (Fig. 1). The attachment of AtrHy lead to an attachment of 9.3 % AtrHy in Pol-OPe-AtrHy, 8.3 % AtrHy in Pol-OPB-AtrHy and 7.7 % AtrHy in Pol-COV-AtrHy and did not significantly change the molecule weight and dispersity of the polymer systems.

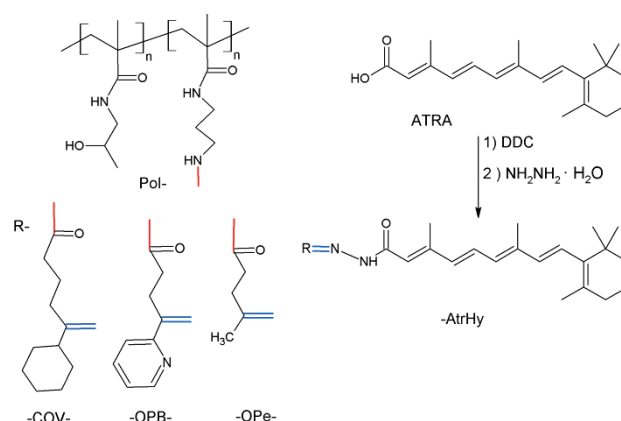


Fig. 1. Schematic structures of polymer conjugates Pol-COV-AtrHy, Pol-OPB-AtrHy, Pol-OPe-AtrHy composed of polymer backbone Pol-; keto acid linkers -COV-, -OPB-, -OPe-; and hydrazide derivative AtrHy.

The hydrodynamic size of the polymer systems in aqueous solution is the crucial parameter for their passive accumulation within the solid tumor, where the EPR effect is observed. We determined the solution behavior of all the polymers using dynamic light scattering. Polymer precursors in aqueous solution formed random coils with a hydrodynamic radius of approximately 4 nm. The hydrodynamic radius of the final polymer conjugates increased almost three times

Table 1. Schematic structures of polymer conjugates Pol-COV-AtrHy, Pol-OPB-AtrHy, Pol-OPE-AtrHy composed of polymer backbone Pol-; keto acid linkers -COV-, -OPB-, -OPE-; and hydrazide derivative AtrHy.

Polymer notation	M_w ($g \cdot mol^{-1}$)	M_w/M_n	R_h (nm)	Linker (type; % mol)	-NH ₂ (% mol)	AtrHy (% wt)
<i>Pol</i>	25870	1.13	4.2	-	-	-
<i>Pol-NH₂</i>	26820	1.11	4.3	-	8.5	-
<i>Pol-OPE</i>	27360	1.17	4.2	OPE; 6.2	0.1	-
<i>Pol-OPB</i>	29390	1.16	3.5	OPB; 7.7	0.0	-
<i>Pol-COV</i>	34770	1.08	3.7	COV; 6.0	0.0	-
<i>Pol-OPE-AtrHy</i> ^{a)}	32900	1.22	10.4	-	-	9.3
<i>Pol-OPB-AtrHy</i> ^{a)}	33920	1.19	12.0	-	-	8.3
<i>Pol-COV-AtrHy</i> ^{a)}	35680	1.10	7.4	-	-	7.7

^{a)} Final polymer-AtrHy conjugate

after attachment of AtrHy (Table 1). We hypothesize that the high hydrophobicity of AtrHy contributes to the amphiphilic character of the conjugate, resulting in aqueous solution in the formation of small aggregates or, more probably, polymer micelles. The increase in R_h seems to be dependent on the combination of the hydrophobicity of AtrHy and the linkage used. The highest R_h was observed for the OPB linkage (12.0 nm) and the smallest for the COV linkage (7.4 nm). A detailed evaluation of the solution behavior of the polymer conjugates is under way.

Release of AtrHy

We selected three keto acids with different structures with the goal of modifying the stability of the hydrazone bond between the AtrHy derivative and the polymer carrier. The stability and rate of AtrHy release were measured under conditions mimicking the pH of human blood (pH 7.4) or the intracellular conditions of lysosomes (pH 5.0). The amount of AtrHy released was calculated by the integration of the areas of UV signal peaks ($A=363$ nm) obtained by HPLC for free (released) and polymer-bound AtrHy. The conjugates containing OPE, COV and OPB keto acid spacers showed different hydrazone bond stability, ranging from very stable (OPB) to less stable (OPE). The key parameter identifying a polymer conjugate as suitable for drug delivery in the organism is its stability in the blood stream. We found that Pol-OPE-AtrHy was less stable at pH 7.4, as almost 50 % of AtrHy was released within 24 h of incubation. In contrast, the polymer conjugates Pol-COV-AtrHy and Pol-OPB-AtrHy seemed to be more stable in the conditions mimicking the blood stream, with 10 % of

AtrHy released from Pol-COV-AtrHy and 2 % from Pol-OPB-AtrHy after 24 hours at pH 7.2 (Fig. 2). A similar release profile was found after 24 h incubation at pH 5.0, the release was the fastest from the OPE-containing conjugate (more than 70 %), moderate for the COV-containing conjugate (50 %) and the lowest for the OPB conjugate (10 %). Thus, the most promising candidate among the three conjugates studied seems to be Pol-COV-AtrHy, which showed high stability in the environment mimicking the blood stream and quite rapid degradation of the hydrazone bond at pH 5.0.

Biological activity

To obtain a polymer conjugate with a pH-sensitive release profile, ATRA had to be modified before attachment to the polymer carrier to its hydrazide derivative AtrHy. The influence of changes in the structure of ATRA (to AtrHy) on the biological properties of the drug was evaluated. The ability of AtrHy and AtrHy bound to the polymer carrier to induce the differentiation of myeloid cells was compared to original ATRA in a proliferation assay using ³H-thymidine incorporation and human HL-60 cells. It is known that these cells strongly downregulate their proliferation during the process of differentiation (Takahashi *et al.* 2014). As expected, the free ATRA showed the strongest capacity to inhibit HL-60 proliferation, whilst AtrHy inhibited the proliferation moderately less. All the conjugates showed lower capacity in inhibiting HL-60 proliferation (Fig. 3). However, this activity remained significant, being the most prominent in the conjugate Pol-OPE-AtrHy and lowest in Pol-OPB-AtrHy. This result is consistent with the release profile of AtrHy, as the release of AtrHy derivative from Pol-OPE-

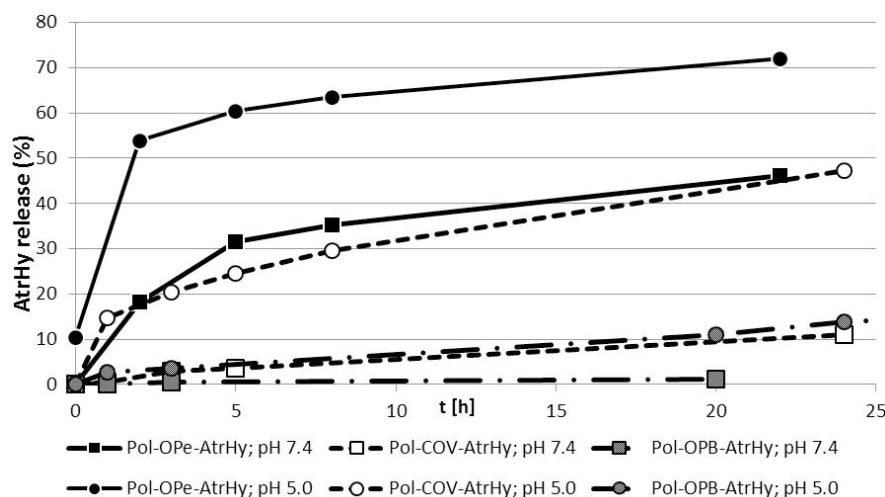


Fig. 2. Release of AtrHy from polymer conjugates. Release kinetics of AtrHy from micellar polymer conjugates: Pol-OPe-AtrHy, Pol-OPB-AtrHy, Pol-COV-AtrHy at pH 5.0 and pH 7.4.

AtrHy is the fastest under both the low pH-simulating conditions in lysosomes (pH 5.0) and in conditions mimicking the blood stream (pH 7.4). However, Pol-OPe-AtrHy releases the ATRA derivatives to a very great extent in the buffer at blood pH; therefore, the COV analog would be more suitable for *in vivo* application. The Pol-COV-AtrHy conjugate exhibited to similar extent activity toward HL-60 cells when compared to the OPe-containing conjugate, whilst its release kinetics was more favorable. No cytostatic respective cytotoxic activity against murine tumor cells, such as T cell lymphoma EL4 or 4T1 carcinoma, was observed using proliferation or metabolic activity assays (data not shown). This fact points to a potential use of the conjugates to modulate the tumor microenvironment rather than to directly dampen the proliferation of tumor growth.

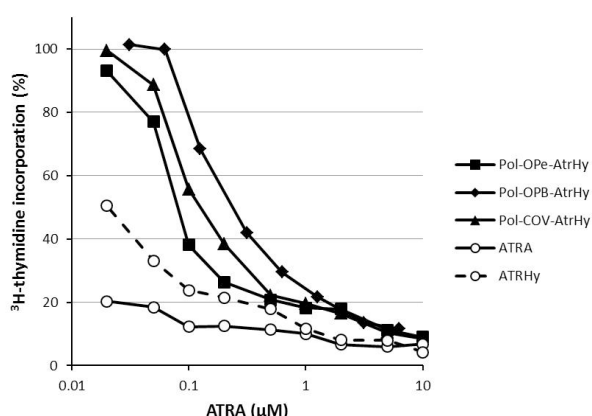


Fig. 3. *In vitro* inhibition of the proliferation of ATRA-responsive HL-60 cells caused by ATRA, AtrHy derivative and AtrHy-containing conjugates. The HL-60 cells were incubated with the polymer conjugates Pol-OPe-AtrHy, Pol-OPB-AtrHy, Pol-COV-AtrHy, with ATRA and AtrHy as controls, at several concentrations. Proliferation was detected by standard ^3H -thymidine incorporation after three days of cultivation.

Conclusions

We have described the design, synthesis and evaluation of the physicochemical characterization and biological activity of several HPMA-based polymer-retinoid conjugates that were tailor-made for immunotherapy of the solid tumors. All the polymer carriers were synthesized using the RAFT polymerization technique to obtain highly defined polymer systems with low dispersity and the ability of passive tumor targeting. In the conjugates, a hydrazide derivative of ATRA was attached to polymer carriers differing in the structure of the side-chain spacer *via* the hydrolytically unstable hydrazone bond. In aqueous solution the polymer-AtrHy conjugates, due to the AtrHy hydrophobicity, formed supra-molecular aggregates or micelles, what can highly support passive accumulation within the solid tumor due to the EPR effect. The conjugates were relatively stable at the pH of blood (7.4) and released active drug under mildly acidic conditions (pH 5.0), a model of the lysosomal environments of cells. The derivatization of ATRA to its hydrazide form slightly decreased its original biological activity, but *in vitro* data proved that AtrHy is still able to slow down the proliferation of HL-60 cells at concentrations which we suppose achievable *in vivo*. Moreover, the polymer-AtrHy conjugates showed decreased but still reasonable capacity to inhibit the proliferation of HL-60 cells. Thus, we can conclude that the polymer-AtrHy conjugate could be a suitable candidate for further *in vivo* studies as an immuno-modulator in solid tumor immunotherapy.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by the Ministry of Education, Youth and Sports of CR within the National

Sustainability Program II (Project BIOCEV-FAR LQ1604).

References

- CHYTIL P, ETRYCH T, KRÍŽ J, SUBR V, ULBRICH K: N-(2-Hydroxypropyl)methacrylamide-based polymer conjugates with pH-controlled activation of doxorubicin for cell-specific or passive tumour targeting. Synthesis by RAFT polymerisation and physicochemical characterisation. *Eur J Pharm Sci* **41**: 473-482, 2010.
- DOLCETTI L, MARIGO I, MANTELLI B, PERANZONI E, ZANOVELLO P, BRONTE V: Myeloid-derived suppressor cell role in tumor-related inflammation. *Cancer Lett* **267**: 216-225, 2008.
- ETRYCH T, CHYTIL P, JELÍNKOVÁ M, ŘÍHOVÁ B, ULBRICH K: Synthesis of HPMA copolymers containing doxorubicin bound via a hydrazone linkage. Effect of spacer on drug release and in vitro cytotoxicity. *Macromol Biosci* **2**: 43-52, 2002.
- ETRYCH T, JELÍNKOVÁ M, ŘÍHOVÁ B, ULBRICH K: New HPMA copolymers containing doxorubicin bound via pH-sensitive linkage: Synthesis and preliminary in vitro and in vivo biological properties. *J Control Release* **73**: 89-102, 2001.
- FANG J, NAKAMURA H, MAEDA H: The EPR effect: Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. *Adv Drug Deliv Rev* **63**: 136-151, 2011.
- FATTAHI A, GOLOZAR MA, VARSHOSAZ J, SADEGHI HM, FATHI M: Preparation and characterization of micelles of oligomeric chitosan linked to all-trans retinoic acid. *Carbohydr Polym* **87**: 1176-1184, 2012.
- GABRILOVICH DI, NAGARAJ S: Myeloid-derived-suppressor cells as regulators of the immune system. *Nat Rev Immunol* **9**: 162-174, 2009.
- JEONG Y IL, SONG JG, KANG SS, RYU HH, LEE YH, CHOI C, SHIN BA, KIM KK, AHN KY, JUNG S: Preparation of poly(DL-lactide-co-glycolide) microspheres encapsulating all-trans retinoic acid. *Int J Pharm* **259**: 79-91, 2003.
- KAPADIA CH, PERRY JL, TIAN S, LUFT JC, DESIMONE JM: Nanoparticulate immunotherapy for cancer. *J Control Release* **219**: 167-180, 2015.
- KOPEČEK J, KOPEČKOVÁ P: HPMA copolymers: Origins, early developments, present, and future. *Adv Drug Deliv Rev* **62**: 122-149, 2010.
- KUSMARTSEV S, CHENG F, YU B, NEFEDOVA Y, SOTOMAYOR E, LUSH R: All-trans-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination. *Cancer Res* **63**: 4441-4449, 2003.
- KUSMARTSEV S, SU Z, HEISER A, DANNULL J, ERUSLANOV E, KÜBLER H, YANCEY D, DAHM P, VIEWEG J: Reversal of myeloid cell-mediated immunosuppression in patients with metastatic renal cell carcinoma. *Clin Cancer Res* **14**: 8270-8278, 2008.
- LIM SJ, LEE MK, KIM CK: Altered chemical and biological activities of all-trans retinoic acid incorporated in solid lipid nanoparticle powders. *J Control Release* **100**: 53-61, 2004.
- MIRZA N, FISHMAN M, FRICKE I, DUNN M, NEUGER AM, FROST TJ, LUSH RM, ANTONIA S, GABRILOVICH DI: All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients. *Cancer Res* **66**: 9299-9307, 2006.
- NEFEDOVA Y, FISHMAN M, SHERMAN S, WANG X, BEG AA, GABRILOVICH DI: Mechanism of all-trans retinoic acid effect on tumor-associated myeloid-derived suppressor cells. *Cancer Res* **67**: 11021-11028, 2007.
- OSTRAND-ROSENBERG S, SINHA P: Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol* **182**: 4499-4506, 2009.
- ŘÍHOVÁ B, ETRYCH T, PECHAR M, JELÍNKOVÁ M, ŠTASTNÝ M, HOVORKA O, KOVÁŘ M, ULBRICH K: Doxorubicin bound to a HPMA copolymer carrier through hydrazone bond is effective also in a cancer cell line with a limited content of lysosomes. *J Control Release* **74**: 225-232, 2001.

- SERAFINI P: Myeloid derived suppressor cells in physiological and pathological conditions: The good, the bad, and the ugly. *Immunol Res* **57**: 172-184, 2013.
- SEYMOUR LW, MIYAMOTO Y, MAEDA H, BRERETON M, STROHALM J, ULBRICH K, DUNCAN R: Influence of molecular weight on passive tumour accumulation of a soluble macromolecular drug carrier. *Eur J Cancer* **31A**: 766-770, 1995.
- SRIVASTAVA MK, ERSSON Å, ZHU L, HARRIS-WHITE M, LEE JM, DUBINETT S, SHARMA S: Myeloid suppressor cells and immune modulation in lung cancer. *Immunotherapy* **4**: 291-304, 2012.
- ŠUBR V, SIVÁK L, KOZIOLOVÁ E, BRAUNOVÁ A, PECHAR M, STROHALM J, KABEŠOVÁ M, ŘÍHOVÁ B, ULBRICH K, KOVÁŘ M: Synthesis of Poly[N-(2-hydroxypropyl)methacrylamide] conjugates of inhibitors of the ABC transporter that overcome multidrug resistance in doxorubicin-resistant P388 cells in vitro. *Biomacromolecules* **15**: 3030-3043, 2014.
- TAKAHASHI H, HATTA Y, IRIYAMA N, HASEGAWA Y, UCHIDA H, NAKAGAWA M, MAKISHIMA M, TAKEUCHI J, TAKEI M: Induced differentiation of human myeloid leukemia cells into M2 macrophages by combined treatment with retinoic acid and 1 α ,25-dihydroxyvitamin D3. *PLoS One* **9**: 1-9, 2014.
- TIWARI MD, MEHRA S, JADHAV S, BELLARE JR: All-trans retinoic acid loaded block copolymer nanoparticles efficiently induce cellular differentiation in HL-60 cells. *Eur J Pharm Sci* **44**: 643-652, 2011.
- UGEL S, DELPOZZO F, DESANTIS G, PAPALINI F, SIMONATO F, SONDA N, ZILIO S, BRONTE V: Therapeutic targeting of myeloid-derived suppressor cells. *Curr Opin Pharmacol* **9**: 470-481, 2009.
- ULBRICH K, ŠUBR V: Structural and chemical aspects of HPMA copolymers as drug carriers. *Adv Drug Deliv Rev* **62**: 150-166, 2010.
- ULBRICH K, ŠUBR V, STROHALM J, PLOCOVÁ D, JELÍNKOVÁ M, ŘÍHOVÁ B: Polymeric drugs based on conjugates of synthetic and natural macromolecules. I. Synthesis and physico-chemical characterisation. *J Control Release* **64**: 63-79, 2000.
- VANNEMAN M, DRANOFF G: Combining immunotherapy and targeted therapies in cancer treatment. *Nat Rev Cancer* **12**: 237-251, 2012.
- WEIDE B, MARTENS A, ZELBA H, STUTZ C, DERHOVANESSIAN E, DI GIACOMO AM, MAIO M, SUCKER A, SCHILLING B, SCHADENDORF D, ET AL.: Myeloid-derived suppressor cells predict survival of patients with advanced melanoma: comparison with regulatory T cells and NY-ESO-1- or melan-A-specific T cells. *Clin Cancer Res* **20**: 1601-1609, 2014.
- WICKI A, WITZIGMANN D, BALASUBRAMANIAN V, HUWYLER J: Nanomedicine in cancer therapy: Challenges, opportunities, and clinical applications. *J Control Release* **200**: 138-157, 2015.
-