Toxicology Letters 274 (2017) 42–50

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Full length article

Cellular response to star-shaped polyacids. Solution behavior and conjugation advantages

Anna Mielańczyk[a], Magdalena Skonieczna[b], Dorota Neugebauer[a,*]

[a] Department of Physical Chemistry and Technology of Polymers, Faculty of Chemistry, Silesian University of Technology, M. Strzody 9, 44-100 Gliwice, Poland
[b] Biosystems Group, Institute of Automatic Control, Faculty of Automatic Control, Electronics and Computer Science, Silesian University of Technology, Akademicka 16, 44-100 Gliwice, Poland

ARTICLE INFO

Keywords:
Week polyacids
Polymer toxicity
Fluorescence
Conjugation

ABSTRACT

The nanosized (~10 nm in 0.01 M PBS and 210 nm in water) star-shaped polymethacrylates with various content of pendant carboxyl groups were characterized *via* basic physicochemical and biological properties toward their use as drug carriers for intravenous administration. The carboxyl groups in polymer were employed to conjugate fluorescein (FA) or doxorubicin (DOX) *via* amide bond formation. In case of DOX, the conjugation efficiency was higher (4.0–16.0%) than of FA conjugation (1.5–4.5%) for corresponding copolymers. The solubility of conjugates strongly depended on the type of attached compound, that is free carriers and their FA conjugates were water-soluble, whereas DOX conjugates were insoluble in water. Cytotoxicity tests performed on model fibroblast and epithelial cell lines showed that negatively charged copolymers (ZP ranged from −75 to −25 mV) were slightly toxic for normal cells (NHDF) and non-toxic for cancer cell lines (HCT-116 and MCF-7/R). The copolymer dose equal to 125 μg/mL resulted in cell viability 118% towards NHDF and 90% for HCT116 cells. The internalization of a representative polymer-fluorescein conjugate by HCT-116 and its accumulation in cytoplasm was proven *via* fluorescence microscopy. MMA/MAA stars showed no adverse effect on HCT-116 cells, hence fluorescein-tagged polymers might be applied as fluorescence probes for *in vitro* imaging, whereas doxorubicin-tagged polymers might be developed as a new polymeric drug carriers.

1. Introduction

The macromolecules as drug carriers are designed in respect to the biocompatibility, non-toxicity, non-immunogenicity and lack of accumulation in the body. Poly(methacrylic acid) (PMAA) is well known from its pH-responsiveness, therefore the conformation and solubility of polymeric chains containing MAA units can be triggered by pH of aqueous media. There is a high probability that the various pH conditions in different tissues and cellular compartments influence on the interactions and self-assembly behavior of the pH-sensitive polymeric systems. Thus, besides size, the surface charge is the most important physicochemical factor affecting DDS in terms of their function in the drug delivery. Generally, the charged particles are taken up by cells better than their uncharged counterparts. It was proven that cells ingest positively charged poly(lactic acid) (Honary and Zahir, 2013; Pillai et al., 2015), chitosan (Yue et al., 2011) and polystyrene (Jiang et al., 2010, 2011) particles in a greater degree than the respective anionic ones. Although positive charge appears to improve the efficacy of drug delivery, a higher cytotoxicity of such nanocarriers has been reported (Capco and Yongsheng, 2014).

The polymeric drug delivery systems (DDS) are often investigated in cooperation with fluorescent organic dyes to monitor the transport pathway by fluorescence microscopy, what can help to improve the effectiveness of delivery (Ding et al., 2014; Wu and Chiu, 2013; Sun et al., 2012). Most of the organic dyes such as fluoresceins, positively charged rhodamines, and cyanines are not used *in vivo* because of their relatively quick photobleaching, which can damage cells and cause substantial cytotoxicity (Han and Burgess, 2009). The same problem concerns fluorescent proteins which additionally suffer from low quantum yield as much as aggregation inside cells causing cellular toxicity (Mauring et al., 2007). Water-soluble semiconducting polymers have demonstrated high sensitivity as biosensors and chemical sensors (Wu et al., 2010). Up till now, these polymers are not degradable and usually contain conjugated phenyl units, which provide poor solubility in water and promote potential carcinogenesis or toxicity in the cases of *in vivo* biomedical applications (Tang et al., 2013; Berezin and Achilefu, 2010). Recently, the fluorescently labeled carbon nano-onions, obtained by a reaction involving the amide bond formation between fluoresceinamine (FA) and the carboxylic groups on the surface, have been applied for *in vitro* imaging of HeLa cells, demonstrating high

* Corresponding author.
E-mail address: dorota.neugebauer@polsl.pl (D. Neugebauer).

http://dx.doi.org/10.1016/j.toxlet.2017.03.022
Received 20 December 2016; Received in revised form 17 March 2017; Accepted 22 March 2017
Available online 24 March 2017

emission properties without significant cytotoxicity (Frasconi et al., 2015). Non-toxicity for HeLa cells was also indicated by the fluorescent labeled Wells–Dawson type polyoxometalates with Sn-organyl derivatives carrying a carboxyl group conjugated with fluoresceinamine, which appeared as randomly dispersed over the entire cytoplasm, or were aggregated in clusters (Geisberger et al., 2013). Another example for alginate–poly-L-lysine-alginate microcapsules labeled with fluoresceinamine were investigated to show the difference in alginate distribution in the Ca-alginate gel beads (Strand et al., 2003).

In this paper, we report the results of preliminary studies on biological properties (cytotoxicity, cellular internalization) of water-soluble star-shape polymethacrylates with salicin core and fluorescently decorated arms as the carriers of FA or DOX. The effect of free carriers, including their sizes and zeta potentials in water/PBS media, on healthy cell line (normal human fibroblast – NHDF) and cancerous cell lines (colon carcinoma – HCT116 as well as breast adenocarcinoma resistant for doxorubicin – MCF-7/R) have been evaluated by means of cytotoxicity assays (MTS and apoptosis assays). The internalization of FA-functionalized copolymers by NHDF as well as HCT116 cells was proved by fluorescence microscopy. We have also described the preparation of DOX-star conjugates *via* amide bonding as the polymeric prodrugs and their general characterization. Unfortunately, the latter conjugates were insoluble in water or phosphate saline buffer (PBS) what disenabled carrying out biological experiments.

2. Materials and methods

2.1. Materials

Fluoresceinamine isomer I (FA, Aldrich, ≥75%), doxorubicin hydrochloride (DOX·HCl, TriMen Chemicals, 97%), 1-ethyl-3-(3-dimethylamionpropyl)-carbodiimide hydrochloride (EDC, Alfa-Aesar, 98%), N-hydroxysuccinimide (NHS, Aldrich, 98%), dimethylsulphoxide (DMSO, Alfa-Aesar, 99%), borax anhydrous (Fluka, ≥98%), 0.1 M sodium phosphate buffer PB (pH = 7.4, prepared by adding appropriate quantity of $NaH_2PO_4xH_2O$ and Na_2HPO_4 to distilled water), 0.01 M phosphate buffer saline (PBS, pH 7.4, Sigma Aldrich), 1.0 M, 4′,6-diamidino-2-phenylindole (DAPI, Sigma Aldrich, 98%), Dulbecco's Modified Eagle's Medium (DMEM, PAN-Biotech) as cell culture media and supplements (Sigma Aldrich), and propidium iodide (PI, Invitrogen) were used as received. Dead Cell Apoptosis Kit with FITC Annexin V (Biomedica) and Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (Promega) containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) were applied in the cytotoxicity studies. Star-shaped $P(MAA\text{-}co\text{-}MMA)_f$ (f = 4 or 6) were synthesized *via* a previously reported procedure (Mielańczyk and Neugebauer, 2014). The procedure of FA and DOX conjugation is described in Supplementary (Scheme 1).

2.2. Methods

2.2.1. Light scattering measurements

The hydrodynamic diameters (D_h) of particle dispersions were measured by dynamic light scattering (DLS) using a Malvern Zetasizer. Samples placed in PMMA cell after dilution with deionized water (0.4 mg/mL) were put in the thermostatted cell compartment of the instrument at 25 ± 0.1 °C. Size measurements were carried out on two samples from five independent runs.

2.2.2. Zeta potential measurements

The surface charges of the polymer matrices were measured in two different media: deionized water and 0.01 M PBS (pH = 7.4). Samples, after dilution (0.04 mg/mL), were placed in disposable folded capillary cell and put in the thermostatted cell compartment of the instrument (Malvern Zetasizer Nano ZS) at 36.6 and 25 ± 0.1 °C. Potential measurements were carried out on two samples from five independent runs. Error bars reported in the ζ-potential plot refer to the width of the ζ-potential distribution for each sample.

2.2.3. Surface morphology

The polymer samples were analyzed by Phenom ProX (Phenom-World B.V.) scanning electron microscope (SEM) in high resolution (10 kV) mode after gold sputtering (5 nm).

2.2.4. Cytotoxicity

Copolymers were tested for cell viability and cytotoxicity using the CellTiter® Aqueous One Solution Cell Proliferation Assay (MTS assay). A total of 10×10^3 NHDF, HCT116 or MCF-7/R cells per well were seeded in a 96-well micro titer plates and incubated overnight at 37 °C to allow cell adherence. A 1 mg/mL stock of star-shaped copolymers prepared in sterile PBS and appropriate volumes of working solution were added to media (DMEM) in wells, resulting 200 μL of the final volume. Cells treated with medium only served as a negative control (blank). Cells were incubated with copolymers at 37 °C in CO_2 for 24 h. After 24 h culturing, the cell culture media was aspirated and washed with PBS. 20 μL of MTS solution was added per well and incubated at 37 °C for 1 h and the absorbance of the formazan product at 490 nm was measured directly from 96-well assay plates using microplate spectrophotometer (BioTekEpoch). All experiments were performed in quadruplicate, and the relative cell proliferation (%) was expressed as a percentage relative to the untreated control cells (positive control).

2.2.5. Cell internalization

Internalization of polymers was observed using fluorescence microscopy and confocal laser scanning microscopy (CLSM). At first, a total of 10×10^3 HCT116 cells per well were seeded in a 96-well micro titer plates and incubated overnight at 37 °C to allow cell adherence. A 1 mg/mL stock of fluorescein labeled star-shape copolymer ($SPAc3^{FA}$) was prepared in sterile PBS and appropriate volumes of working solution were added to media already in wells resulting 200 μL of DMEM media and respective polymer. Cells were incubated with $SPAc3^{FA}$ at 37 °C in CO_2 for 24 and 48 h. After culturing, the cell culture media was aspirated and washed with PBS. After staining with DAPI the cells were examined by fluorescence microscopy (Olympus xcellence rt).

A total of 500 μL/well of 10×10^3 of HCT116 cells were seeded directly onto a 4-well culture slides and incubated for 24 h. Thereafter, the media was aspired and 300 μL of fresh DMEM and polymer solution were added resulting in 500 μg/mL of polymer final content. The cells were incubated at 37 °C for 24 h. The media was aspirated and the remaining attached cells were washed with PBS and fixed with 70% ethanol for 10 min followed by washing with deionized H_2O. After staining with DAPI, the slides with cells were examined by CLSM (Olympus FV1000 microscope). All imaging conditions, including laser power, photomultiplier tube, and offset settings, were aligned with fluorescence intensity of sample.

2.2.6. Apoptosis analysis by flow cytometry

A total of 5×10^4 HCT116 cells were suspended in 980 μL of DMEM media, and were incubated with 20 μL of a 250 μg/mL of star copolymers in a 12-well culture plate. The cells were incubated with the star polymers for 24 h at 37 °C. The control group did not receive any polymers. Then the cells were collected into 1.5 mL tubes and centrifuged at 1100 rpm for 3 min to pellet the cells. The supernatant was removed and the cells were washed with 200 μL of PBS followed by centrifugation at 1100 rpm for 3 min. The cells were resuspended in Annexin V Binding Buffer and transferred to a borosilicate tubes followed by 2.5 μL of FITC Annexin V addition. After 15 min the 250 μL of PBS and 10 μL of PI solution (100 μg/mL) were added. The cells were gently vortexed and incubated for 15 min at r.t. in the dark. Each sample was analyzed using a BD ARIA III cytometer.