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A new generation of biocompatible nanoparticles made of resorbable poly(ester amide)s

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A B S T R A C T

A new generation of resorbable nanoparticles (NPs) were prepared on the basis of amino acid based biodegradable (AABB) poly(ester amide)s (PEAs) for drug delivery application. The NPs were fabricated by cost-effective polymer deposition/solvent displacement (nanoprecipitation) method on the basis of three different AABB PEAs recently developed by our group: (i) PEA composed of amino acid leucine as a basic component, (ii) cationic PEA composed of amino acid arginine for imparting positive charge, and (iii) functional PEA composed of amino acid leucine and lateral poly(ethylene glycol) groups acting as surfactant as well as PEGylating agent. The mean particle diameter (MPD), polydispersity index (PDI) and zeta-potential (ZP) were determined by Dynamic Light Scattering (DLS). Moreover, the stability (resuspendability) of the NPs over the time at low temperature was investigated. The NPs were studied for in vitro cell compatibility using four different stable cell lines: A549 (human), U937 (human), RAW264.7 (murine), Hepa 1-6 (murine). Prepared nanoparticles exhibit high stability and cell compatibility and have potential for the application as drug delivery devices.

Keywords: Biodegradable polymers; Nanoprecipitation; Nanoparticles; Biodegradable surfactant; PEGylation; in vitro cell compatibility

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Introduction

There is increasing interest to develop new nanoscale drug delivery vehicles for targeted therapy [1-3]. Targeted drug delivery compared to conventional one, has potential to increase delivery efficacy and reduce side effects. Nanoparticles (NPs) used as nanocontainers should meet following requirements to be suitable for drug delivery: high particle stability, tunable carrier capacity, feasibility of encapsulation of both hydrophilic and hydrophobic drugs, feasibility of variable routes of administration, including oral application and inhalation, and ability to allow controlled drug release from the matrix. Moreover, it is highly desirable to prepare nanoparticles without surfactants, because the surfactant residue is difficult to remove and can cause toxicity, affect drug delivery and cellular uptake efficiency [4-6].

Among various types of drug nanocarriers, biodegradable polymer based nanoparticles emerged as one of the most promising class, as they have ability safely to be cleared from the body after the fulfillment of their purpose [7]. Number of synthetic biodegradable polyesters of low immunogenicity such as poly(caprolactone), poly(lactic acid) and poly(lactic-co-glycolic acid) were reported as suitable candidates for design of nanocarriers [8].

However, degradation products of these polymers are glycolic and lactic acids with pKa 3.83 and 3.86, accordingly, that are considered to be toxic and induce undesired phenotype modulation in cells [9]. Besides, polyesters showed lower affinity to living tissues (due to the lack of hydrophilic CO–NH bonds in the backbone) [10] that can decrease the bioavailability of the NPs prepared from this type of polymers. Therefore, biodegradable poly(ester amide)s (PEAs) are considered as better candidates for biomedical applications, as they contain in the backbone CO–NH links along with ester bonds leading to an increased polymer-tissue affinity. The most promising are the PEAs composed of physiological building blocks − naturally occurring α-amino acids and other non-toxic building blocks such as fatty diols and dicarboxylic acids - amino-acid-based biodegradable (AABB) polymers [9,11–19]. The AABB polymers showed better biocompatibility compared to polyesters [9,15–17]. Besides, after the biodegradation of the AABB polymers very low or no local acidic environment causing inflammation is formed [20]. It has to be emphasized that the AABB PEAs were successfully used for constructing nanobiocomposites [21,22], drug eluting vascular stent coatings [19,23–25], microfibrils [26] and microspheres [27].

One of the main factors limiting the application of NPs is the problem of so-called protein "corona", that is conjugated with the immune system of the organism. When nanosystems are in a physiological environment, they rapidly adsorb biomolecules such as proteins and lipids on their surface forming a protein corona [28,29]. Therefore, in addition to size, shape, and other nanoscale parameters of the nanomaterial, the long-lived (hard) corona has an important impact on the behavior of NPs in biological media. The formation of protein corona changes the size, surface chemistry, solubility, aggregation, and surface charge of the NPs and hence can influence their biodistribution, cellular uptake, and capture by macrophages. In other words, the therapeutic potentialities of polymeric NPs may be compromised by particle recognition by the macrophages [28,29]. It has been established that the NPs functionalized with hydrophilic polymers (NPs having so called stealth coatings [28]) show more long-lasting circulation and decreased macrophage recognition of many types of nanoparticles. One of the efficient ways to render the NPs surface hydrophilic is their PEGylation which represent the process of pretreatment of polyethylene glycol (PEG) on the surface of NPs. PEG decreases the affinity of plasma proteins (opsonins) for adsorption on NPs - long chains of PEG form a random cloud around the NPs, thereby preventing absorption of opsonins and in that way suppressing phagocytosis. Along with the protection of the NPs from phagocytosis the PEGylation substantially increases the bioavailability of NPs [28−33].

Positive surface charge (positive zeta-potential) is favourable for penetration of biological barriers, including ophthalmic ones, such as cornea, lens, etc. It is known that a positive charge helps with the NPs' adhesion to the surface of cells and stimulates penetration into the cells via endocytosis [30,31].

Recently we reported on a systematic study of the preparation of resorbable NPs by cost-effective nanoprecipitation method using AABB PEAs [34]. The present work deals with the preparation and study of the new generation of modified NPs having the PEGylated and positively charged surfaces. The study also includes the cell compatibility assessment of the new NPs with four established cell lines.

1. Materials and Methods

1.1. Materials

Surfactants − Tween 20 Sorbitanmonolaurate (MW 1,228), Tween 40 Sorbitanmonopalmitate (MW 1,277), Tween 80 Sorbitanmonooleate (MW 1,310), Kolliphor P188 PPO-PEO-PPO triblock copolymer (MW, 7,680-9,510), Brij 010 Polyoxyethylene(10) oleyl ether (MW 709), Poly(vinyl alcohol)s (PVAs) such as Mowiol 4-88 (MW 31,000 of 86.7%–88.7% hydrolyzed) and Mowiol 8-88 (MW 67,000 of 86.7–88.7% hydrolyzed), purchased from Sigma-Aldrich (St. Louis, MO, USA), and Ttriton X100 Poly(ethylene glycol)p-(1,1,3,3-tetramethyl-butyl)-phenyl ether (MW 647) purchased from Ferak Berlin GmbH (Berlin, Germany), were used as received. Methoxy-PEG-amine with average molecular weight 2,000 Da (mPEG-amine-2000) was purchased from Laysan Bio. Organic solvents − N,N-Dimethylformamide (DMF) and N,N-dimethylacetamide (DMA) were purchased from Sigma-Aldrich, and Dimethylsulfoxide (DMSO) from Carl Roth (Karlsruhe, Germany). All the solvents were used as received. The dialysis bag

(MWCO 25 kDa) was purchased from Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA. The AABB PEAs, selected for the proposed study (Fig. 1), were originally synthesized as reported previously – the leucine (L) based PEA 8L6 via the Interfacial Polycondensation (IP) [15,20], and the arginine (R) based biodegradable cationic PEA 8R6 - via Solution Active Polycondensation (SAP) [35]. Na2EDTA (Ethylenediaminetetraacetate disodium salt) solution (Santa Cruz Biotechnology, Dallas, USA) has been used. One of the used cell lines - U-937, grows in suspension. This particular cell line was maintained in RPMI1640 medium (Santa Cruz Biotechnology, Dallas, USA) supplemented with 10% FBS.

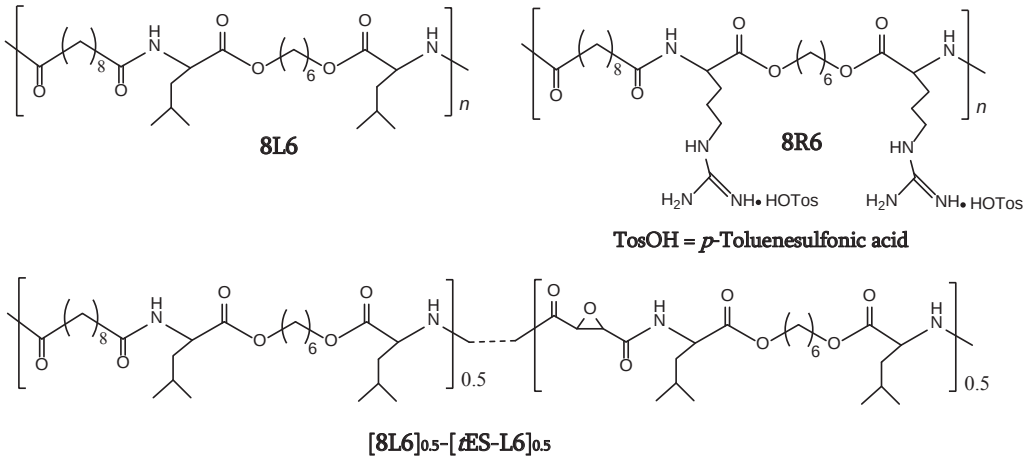

Fig. 1.*The chemical structures of the PEAs used for NPs fabrication: PEA 8L6, cationic PEA 8R6, and epoxy-co-PEA [8L6]$_{0.5}$-[tES-L6]$_{0.5}$ - a precursor of the new surfactant/ PEGylating agent PEG-co-PEA.*

The new surfactant/PEGylating agent composed of amino acid L and containing lateral PEG chains, PEG-co-PEA, was synthesized by interaction of epoxy-co-PEA [8L6]0.5-[tES-L6]0.5 with mPEG-amine-2000; the epoxy-precursor [8L6]0.5-[tES-L6]0.5 was synthesized via SAP as reported previously [36].

Four different established cell lines (two - human, two - murine) were used: A549 − human alveolar epithelial type II cells derived from lung carcinoma (ATCC® CCL-185TM), U-937 – human monocytic cell line from histiocytic lymphoma (U-937 ATCC CRL-1593.2TM , city, US state, USA), Hepa1-6 − mouse hepatoma-derived cells (ATCC®CRL-1830TM), and RAW264.7 − mouse leukemic monocyte macrophage cell line (ATCC®TIB-71TM). Three cell lines (A549, Hepa 1-6, and RAW264.7) are adherent and have been maintained in complete Dulbecco's Modified Eagle's Medium(DMEM) Santa Cruz Biotechnology, Dallas, USA) culture medium supplemented with 10% fetal bovine serum(FBS) Santa Cruz Biotechnology, Dallas, USA). For the harvesting trypsin (0.25%)/

1.2. Characterization of polymers

The number-average (Mn), and weight-average (Mw) molecular weights (MW), and molecular weight distribution (MWD) of the polymers were determined using the GPC. The MWs of the polymers 8L6 and [8L6]0.5-[tES-L6]0.5 were determined on a machine of Waters Associates, Inc., Milford, MA, USA, equipped with Styragel columns in DMF: HR4, HR3, HR0.5 (all 7.8 mm × 300 mm), a high-pressure liquid chromatography pump (Waters 1525 Binary HPLC) and a Waters refractive index detector 2414 and UV-detector (Waters 2487 dual absorbance detector, λ = 240 nm). A solution of LiBr (0.1 M) in DMF was used as an eluent. Injected volume was 100 μL, the sample concentration 5.0 mg/mL, flow rate 1.0 mL/min and temperature 35 °C. The columns were calibrated with PMMA standards. The MW of the cationic polymer 8R6 was determined on a Shimadzu GPC machine, model LC-8A equipped with an Empower computer program (Waters), a PL HFIP gel column (Polymer Lab, Theale, Berkshire, UK)

and a refractive index detector (Shimadzu RID-10A, Shimadzu Scientific Instruments, Columbia, MD, USA). The polymer 8R6 was dissolved in and eluted with HFIP containing CF3COONa (0.05 M, to suppress polyelectrolyte effects). The injected volume was 100 μL, the sample concentration 2.0 mg/mL, and the flow rate 0.5 mL/min. The columns were calibrated with PMMA standards.

The [8L6]0.5-[tES-L6]0.5 and PEG-co-PEA co-polymers were also characterized by FT-IR and 1H and 13C NMR spectroscopy. Thermo Nicolet Avatar 370 FT-IR spectrophotometer (coupled with EZ OMNIC software) was used for IR analysis. To obtain the polymers spectra the thin films were cast from dichloromethane solution on KBr plates, solvent was evaporated at r.t., and films were dried in a vacuum at 40° C for 24 h. The 1H and 13C NMR spectra were recorded using JEOL ECA-400 MHz NMR spectrometer at r.t. in DMSO-d6 as a solvent and internal standard.

1.3. Preparation of PEG-co-PEA

For PEGylating NPs we have originally developed a new biodegradable PEGylating agent which at the same time represents a surfactant. The new surfactant/PEGylating agent - PEG-co-PEA was synthesized via polymeranalogues modification reaction: 200 mg (0.42 mmol) of $[8L6]_{0.5}$-$[t$ES-L6$]_{0.5}$ and 840 mg (0.42 mmol) of mPEG-amine-2000 was dissolved in 2 mL DMA and stirred for 24 h at 60°C. After completing the reaction, the resulting solution was poured in 50 mL hexane and the precipitated product was separated and dried under reduced pressure at 60°C for 48 h.

The structures of initial co-polymer $[8L6]_{0.5}$-$[t$ES-L6$]_{0.5}$ and the obtained PEG-co-PEA were confirmed by FTIR and NMR spectroscopy.

$[8L6]_{0.5}$-[tES-L6]$_{0.5}$. ^{1}H NMR (400 MHz, DMSO-d6, δ): 0.9 (24H, s, CH(C$\underline{H}_3$)$_2$), 1.1-1.84 (40H, O-CH$_2$-(C$\underline{H}_2$)$_n$-, C$\underline{H}$(CH$_3$)$_2$ and –CH-C$\underline{H}_2$-CH-), 2.05 (4H, t, -CO-C$\underline{H}_2$), 3.60 (2H, s, C$\underline{H}$-O epoxy), 4.04 (8H, -O-C$\underline{H}_2$-), 4.27 (4H, -NH-C$\underline{H}$-CO), 8.1 (2H, d, CH$_2$-CO-N$\underline{H}$-CH-), 8.82 (2H, d, O-CO-N$\underline{H}$-CH- and O-CH-CO-N$\underline{H}$-CH-).

^{13}C NMR (100 MHz, DMSO-d6, δ): 23.0, 24.6, 25.2, 28.3, 29.03, 46.1, 50.8, 52.7, 64.7, 166.1, 172.0, 172.5, 172.8.

PEG-co-PEA. ^{1}H NMR (400 MHz, DMSO-d6, δ): 0.88 (24H, s, CH(C$\underline{H}_3$)$_2$), 1.12-1.84 (40H, O-CH$_2$-(C$\underline{H}_2$)$_n$-, C$\underline{H}$(CH$_3$)$_2$ and –CH-C$\underline{H}_2$-CH-), 2.09 (4H, t, -CO-C$\underline{H}_2$), 3.51 (PEG -O-CH$_2$-CH$_2$-O-), 3.60 (1H, C(OH)-C$\underline{H}$), 4.04 (8H, -O-C$\underline{H}_2$-), 4.24 (4H, -NH-C$\underline{H}$-CO), 4.62 (1H, C(OH)-C$\underline{H}$), 7.90-8.35 (5H, N$\underline{H}$). Together with ^{1}H NMR spectroscopy, successful modification of epoxy group with PEG-amine was proved by complete disappearance of the epoxide band at 895 and 3062 cm^{-1} combined with appearance of the broad band at ≈ 3500 cm^{-1} characteristic for OH group (signal is partially overlapped with absorption band of amide at 3302 cm^{-1}).

2.4. Preparation of the PEGylated NPs

The PEA NPs were prepared according to the polymer deposition/solvent displacement (nanoprecipitation) method under the optimal conditions previously established for amino acid based biodegradable ester polymers [34]: 6.0 mg of PEA 8L6 was dissolved in 1.0 mL of DMSO (organic phase) and dropwise added (dropping rate 12 drops/min) to 10.0 mL of water (inorganic phase) containing 50.0 mg of the initially synthesized biodegradable surfactant PEG-co-PEA (organic/water phases (O/W) ratio 1:10 v/v) at a stirring rate of 700 rpm using a magnetic stirrer. All manipulations were done at room temperature.

PEGylated NPs were also fabricated using the modified nanoprecipitation method as reported previously [34]: the half of the surfactant PEG-co-PEA (25.0 mg) was dissolved in 1.0 mL of DMSO together with 6.0 mg 8L6, or the 70/30 mixture of 8L6/8R6 (organic phase) and dropwise added to 10.0 mL of water phase containing the other half of the surfactant PEG-co-PEA (25.0 mg), i.e. in this method the surfactant is equally distributed in both organic and water phases.

In all cases, after adding the organic phase, the aqueous phase became turbid indicating formation of NPs. The suspensions of the NPs, obtained after the complete addition of the organic phase, were stirred for 10-15 min and then dialyzed against distilled water for 1 h using the dialysis bag with MWCO 25 kDa to remove the organic solvent and residual surfactant. After dialysis the volume of suspension was reduced to 10.0 mL by evaporating water on a rotary evaporator under reduced pressure. The obtained nanosuspensions were stored at 4-5°C.

2.5. NPs size, size distribution and zeta-potential

The obtained PEGylated NPs were characterized by size (Mean Particle Diameter - MPD), size distribution (Polydispersity Index - PDI), and zeta-potential (ZP), which were determined by dynamic light scattering (DLS) using a particle size analyzer (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK) at 25 °C. The MPD and PDI are presented as an average of five measurements ± standard deviation (SD). The PDI < 0.04 corresponds to a narrow distribution, 0.04 ≤ PDI ≤ 0.16 − to a mean distribution, and PDI > 0.16 − to a wide distribution.

2.6. Cytotoxicity (MTT) assay

For the cytotoxicity testing of A549, U-937, Hepa1-6, and RAW264.7, cells were cultured at a density 0.5 × 106 cell/mL in 96-well cell microplates. After 24–28 h of growth, after 80% confluence has been reached, the cell culture medium was changed to serum-free and nanoparticles were added at a concentration of 5.0 μg/mL. After 24 h, the cytotoxicity of NPs was assessed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay [37] as described by us previously [28]. The absorbance at a wavelength of 570 nm was read on the ELx800 Absorbance Reader (Biotek). In control samples cells were cultured with medium only. Cell viability was calculated using the equation:

where [OD]test, [OD]control, and [OD]blank represented the absorbance values of the wells with cells and NPs, cells without NPs, and without NPs and cells, respectively. For each experiment the absorbance was the average value measured from 12 wells in parallel. Five independent experiments have been performed in case of each cell line used.

$$\text{Cell viability (\%)} = \frac{([OD]\text{test}-[OD]\text{blank})}{([OD]\text{control}-[OD]\text{blank})} \times 100\% \qquad (1)$$

2. Results and discussion

2.1. Selection of the PEAs

The PEA 8L6 composed of L-leucine (L), 1,6-hexanediol (6) and sebacic acid (8) was selected as a basic polymer for preparing the PEGylated NPs. We have found this PEA as an optimal for fabricating resorbable NPs considering such parametes as stability upon storage and cell compatibility [34]. For imparting a positive charge to the NPs enhancing both their stability and cellular uptake [30,31], arginine-based cationic PEA 8R6 was used. Among designed arginine-based PEAs [35] 8R6 showed a high hydrophobicity - it dissolves in water only upon heating to 60–70 °C and precipitates when cooled to r.t. We have assumed it will be retained by the NPs, i.e. will not easily be washed out from the NPs in the water phase. The third polymer we have selected in the present work was the functional epoxy-co-PEA [8L6]0.5-[tES-L6]0.5 containing reactive oxirane rings. It was demonstrated [36] that the oxirane rings are suitable active sites for covalent binding to primary amines under mild conditions. This approach was used for preparing the new amphiphilic polymer by reacting [8L6]0.5-[tES-L6]0.5 with mPEG-amine-2000. The new amphiphilic copolymer labeled as PEG-co-PEA combines the properties of both surfactant and PEGylating agent: the copolymer contains backbone similar to the backbones of 8L6 and 8R6 that provides a high affinity between these polymers, that in turn, should provide a firm anchoring of the PEG-co-PEA with NPs made of the 8L6 or 8L6/8R6. The structures of the selected PEAs are depicted in Fig. 1, their MWs are given in Table 1.

Table 1. *MW characteristics of the PEAs*

Polymer	M_w	M_n	M_w/M_n
8L6	76,100	44,200	1.72
8R6	17,500	7,200	2.43
$[8L6]_{0.5}$-$[tES\text{-}L6]_{0.5}$	27,200	14,700	1.85
PEG-co-PEA	36,800	28,400	2.58

3.2. Fabrication of the PEGylated NPs

As it was mentioned above, for PEGylation of NPs we have initially prepared the new biodegradable amphiphilic polymer PEG-co-PEA, which at the same time serves as a surfactant when preparing the NPs. In contrast to its precursor - epoxy-co-PEA [8L6]0.5-[tES-L6]0.5 the new amphiphilic polymer is soluble in water and similar to the known amphiphilic compounds (surfactants) forms micelles (Table 2). The mean particle diameter (MPD) of the PEG-co-PEA micelles is 16.2 nm that is close to MPD of the micelles formed by Brij 010 and Mowiols. As regards the zeta-potential (ZP), there is no significant difference between the PEG-co-PEA micelles (ZP=-13.1 mV) and micelles of known surfactants (Table 2).

A high affinity of the backbones of the PEG-co-PEA, 8L6 and 8R6 provided effective conjugation of PEG-co-PEA with the surface of the NPs and promoted the fabrication of the NPs (Table 3). The results given in Table 3 show that the MPD of the negatively charged 8L6 NPs is less than the MPD of the positively charged 8L6/8R6 NPs. Thus, the MPD of 8L6 NPs is 70.1 nm (in case of nanoprecipitation method, NM) and 97.6 nm (in case of modified nanoprecipitation method, MNM) vs. 125.7 nm and 130.2 nm obtained for 8L6/8R6 NPs.

Table 2. *Characteristics of micelles of standard surfactants and new biodegradable surfactant*

Surfactant	MPD (nm) ± SD	PDI ± SD	Z
Tween 20	11.3 ± 0.3	0.244 ± 0.029	
Tween 40	11.1 ± 0.8	0.231 ± 0.023	
Tween 80	10.8 ± 0.7	0.263 ± 0.018	
Brij010	19.2 ± 0.3	0.173 ± 0.016	
Kolliphor P188	9.2 ± 0.4	0.373 ± 0.031	
Triton X-100	10.4 ± 0.7	0.254 ± 0.021	
Mowiol 4-88	20.0 ± 1.3	0.463 ± 0.039	
Mowiol 8-88	23.4 ± 1.5	0.479 ± 0.041	
PEG-co-PEA	16.2 ±1.0	0.174 ± 0.012	

Table 3. *Characteristics of 8L6 and 8L6/8R6 PEGylated NPs prepared by nanoprecipitation (NM) and modified nanoprecipitation methods (MNM)*

Method	MPD (nm) ± SD	PDI ± SD	ZP (mV) ± SD
8L6 NPs			
NM	70.1 ± 2.3	0.188 ± 0.002	-14.5 ± 1.2
MNM	97.6 ± 2.6	0.112 ± 0.008	-14.7 ± 1.1
8L6/8R6 (70/30) NPs			
NM	125.7 ± 4.3	0.221 ± 0.014	+6.9 ± 1.2
MNM	130.2 ± 3.8	0.143 ± 0.011	+7.5 ± 0.4

As we can see from Table 3, obtained PEGylated NPs showed mean ($0.04 \leq PDI \leq 0.16$) to wide size distribution ($PDI > 0.16$). It has to be noted that in case of MNM for both 8L6 and 8L6/8R6 NPs the size distribution was mean − 0.112 and 0.143 and in case of NM it was wide - 0.188 and 0.221, accordingly. With regard to the surface charge two types of NPs were prepared: (i) negatively charged NPs based on PEA 8L6 and (ii) positively charged NPs based on the mixture 8L6/8R6 (70:30 w/w). The ZPs of the NPs, listed in Table 3, were determined right after the fabrication of the NPs. The results show that the PEGylated 8L6 NPs had moderate negative charge: -14.5 mV in case of NM and -14.7 mV in case of MNM. We suppose that the negative ZP of the NPs is caused by a partial hydrolysis of the ester links of the PEAs generating free carboxyl groups (carboxylate anions –COO−). In case of the NPs prepared from 8L6/8R6 mixture, the ZP values were positive for both applied methods; +6.9 mV (NM) and +7.5 mV (MNM). The positive charge of the 8L6/8R6 NPs is provided by guanidine groups of the cationic PEA - 8R6. In spite of relatively low surface charge value of the 8L6/8R6 NPs, it is sufficient to ensure the stability upon storage (see below).

3.3. Stability of the NPs

A firm anchoring of the PEG-co-PEA with NPs made of the 8L6 or 8L6/8R6 provides good stabilization of the NPs. Both types of the PEGylated NPs prepared by NM and MNM were studied for stability upon storage at low temperature. The NPs' MPD and PDI were measured right after the fabrication and then the NPs suspensions were stored refrigerated at 4–5 °C. After predetermined time (30, 60, and 90 days), the suspensions were thoroughly shaken and analyzed for the MPD and PDI. The results, listed in Table 4, show that the fabricated NPs were highly stable – no substantial change of the MPD and PDI, or aggregation is observed after 90 days of storage.

3.4. Cell compatibility study of the NPs

For cell compatibility studies we have used established cell lines of different origin: two murine cell lines (Hepa1-6 and RAW264.7) and two human cell lines (A549, U-937). In case of both species one cell line was monocytic (RAW264.7, U-937). We have chosen monocyte-macrophage cell lines for the cytotoxicity studies, as these particular cells are characterized by high phagocytic activity. Therefore, presumably, these cells will actively phagocytose added NPs, which will lead to the higher concentration of NPs inside the cells, compared to the cell types which aren't able to perform phagocytosis and will engulf NPs only by the process of endocytosis. Our aim was to investigate how the high concentration of intracellular NPs inside the phagocytes will affect cell viability. In parallel, we have taken a non-phagocytic cell line for each of the species: hepatocytes (Hepa1-6) - in case of mouse and alveolar epithelial type II cells (A549) - in case of human. For the viability assessment standard MTT test has been used, which is based on the ability of a mitochondrial dehydrogenase enzyme in viable cells to cleave the tetrazolium salt leading to coloured reaction [37]. As it can be seen from the Fig. 2, both types of NPs haven't affected cell viability in case of all four cell lines used: no statistically significant change of the cell viability is visible compared to subsequent control cultures. This means that both types of NPs are characterized by high biocompatibility. Considering the fact, that in cultured cell lines NPs might affect other physiological parameters besides viability, in our future experiments we plan to evaluate NPs effect on cells growth and functional characteristics.

Table 3. *The stability of the prepared NPs upon storage at 4-5°C.*

Type of NPs	Method	Time			
		Freshly prepared	After 30 days	After 60 days	After 90 days
		MPD (nm) ± SD [PDI ± SD]			
8L6 NPs	NM	70.1 ± 2.3 [0.188 ± 0.002]	72.2 ± 1.3 [0.181 ± 0.006]	70.4 ± 1.9 [0.179 ± 0.005]	71.8 ± 2.3 [0.178 ± 0.009]
	MNM	97.6 ± 2.6 [0.112 ± 0.008]	99.2 ± 3.2 [0.119 ± 0.006]	95.8 ± 3.4 [0.129 ± 0.012]	98.3 ± 2.8 [0.121 ± 0.011]
8L6/8R6 NPs	NM	125.7 ± 4.3 [0.221 ± 0.014]	118.4 ± 5.1 [0.229 ± 0.012]	121.9 ± 3.8 [0.219 ± 0.009]	119.2 ± 4.1 [0.218 ± 0.006]
	MNM	130.2 ± 3.8 [0.143 ± 0.011]	131.4 ± 3.1 [0.151 ± 0.016]	128.7 ± 4.2 [0.140 ± 0.009]	129.3± 4.4 [0.136 ± 0.010]

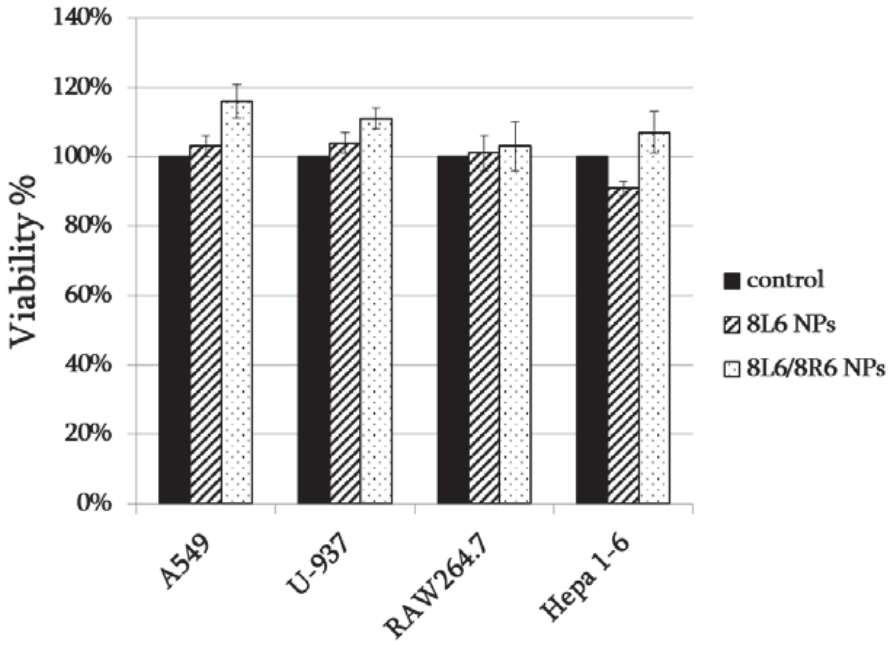

Fig. 2. *Percentage of viable cells after 24 h incubation with 8L6 and 8L6/8R6 NPs. Results for four cell lines shown: A549 – human alveolar epithelial type II cells derived from lung carcinoma, U-937 – human monocytic cell line from histiocytic lymphoma, Hepa1-6 – mouse hepatoma-derived cells, and RAW264.7 –* mouse leukemic monocyte macrophage cell line.

3. Conclusion

Two types of PEGylated NPs (negatively and positively charged) on the basis of AABB PEAs were prepared using cost-effective nanoprecipitation and modified nanoprecipitation methods. For preparing NPs new biodegradable surfactant/PEGylating agent was specially designed via polymeranalogues modification reaction. The stability (resuspendability) of the PEGylated NPs upon storage was investigated using DLS method. *In vitro* biocompatibility study of the NPs with four different stable cell lines: A549 (human), U-937 (human), RAW264.7 (murine), Hepa 1-6 (murine) showed that they are biocompatible. Considering the high stability and biocompatibility, prepared NPs are considered as promising candidates for the application as drug delivery nanocarriers.

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