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Formation and characterization of DNA-polymer-condensates based on poly(2-methyl-2-oxazoline) grafted poly(L-lysine) for non-viral delivery of therapeutic DNA

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ABSTRACT

Successful gene delivery systems deliver DNA in a controlled manner combined with minimal toxicity and high transfection efficiency. Here we investigated 15 different copolymers of poly(L-lysine)-*graft*-poly(2-methyl-2-oxazoline) (PLL-*g*-PMOXA) of variable grafting densities and PMOXA molecular weights for their potential to complex and deliver plasmid DNA. $PLL_{20}g_7PMOXA_4$ formed at N/P charge ratio of 3.125 was found to transfect 9 ± 1.6% of COS-7 cells without impairment of cell viability. Furthermore these PLL-g-PMOXA-DNA condensates were internalized 2 h after transfection and localized in the perinuclear region after 6 h. The condensates displayed a hydrodynamic diameter of ~100 nm and were found to be stable in serum and after 70 °C heat treatment, moreover the condensates protected DNA against DNase-I digestion. The findings suggest that DNA-PMOXA-g-PLL condensate formation for efficient DNA-delivery strongly depends on PMOXA grafting density and molecular weight showing an optimum at low grafting density between 7 and 14% and medium N/P charge ratio (3.125–6.25). Thus, $PLL_{20}g_7PMOXA_4$ copolymers might be promising as alternative to PLL-g-PEG-DNA condensates for delivery of therapeutic DNA.

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1. Introduction

Controlled delivery of therapeutic genes becomes more and more important in today's medicine. Especially for diseases such as dystrophies, cancer or deficient wound healing in diabetes patients, gene therapy might be an important clinical option [1–8]. Non-viral delivery, which is considered safer and simpler to handle and more cost effective compared to viral systems, include gene delivery by either naked DNA, lipid-based (lipoplexes) or by polycationic molecules [6,9–14].

Predominantly used polycationic polymers comprise poly(L-Lysine) (PLL), polyethylenimine (PEI), poly-L-ornithin and chitosan as they are positively charged under physiological conditions and therefore condense negatively charged DNA efficiently [12,14–16]. Unfortunately, undecorated polycationic polymers were shown to enhance membrane permeability and induce nanoscale pore formation combined with high cytotoxicity [12,17]. For this reason cationic polymers were grafted with bioinert poly(ethylene glycol) (PEG), in order to mask cationic charge, to prevent aggregation and to increase cellular uptake and stability [18–23]. Condensates formed by low PEGylated PLL and plasmid DNA were recently shown to hold excellent transfection efficiencies combined with low cytotoxicity in COS-7 cells [5,24,25].

Although PEG is by far the most frequently used bioinert polymer, PEG's ether groups are prone to undergo oxidative degradation in aqueous solutions. Moreover, PEG coatings have been shown to lose their function in *in vivo* applications very fast and were shown to induce hypersensitivity in certain patients [26–29]. Therefore alternatives to PEG-based structures are highly demanded. Hence, protein-repellent surface coatings based on highly bioinert poly(2-methyl-2 oxazoline) (PMOXA) were developed [30–35]. PMOXA decorated liposomes were previously shown to have long circulation times in blood [36,37], and vesicles consisting

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of amphiphilic PMOXA terminal triblock copolymers allowed the incorporation of functional channel-forming membrane proteins [38]. Interestingly, nanocontainers formed of hydrophilic PMOXA terminal triblock copolymers did not activate human monocytic THP-1 cells and showed very low nonspecific binding to the cell surface [39]. Therefore PMOXA has attracted interest as alternative to PEG in biological- and biomedical applications. PMOXA is composed of a poly-ethylene-imine backbone with amide-bonded pendent acetyl groups. For this reason PMOXA resembles PEG in having a heteroatom separated by an ethylene unit in its backbone (Fig. 1) [33,34]. In contrast to PEG, PMOXA offers a peptide-like structure due to its isomerism to poly(homo-alanin) and is suspected to resist auto-oxidation and might therefore be a more stable bioinert polymer.

In this study, different PLL-g-PMOXA polymers are explored as gene delivery vehicles for therapeutic DNA. The influence of PMOXA's molecular weight and the degree of PMOXA grafting on transfection efficiency and cell viability in COS-7 cells was investigated. Furthermore, stability of PLL-g-PMOXA-DNA condensates with respect to temperature, serum components and DNase-I digestion was studied, being fundamental for storage of preformed DNA-polymer condensates followed by delivery of therapeutic DNA.

2. Materials and methods

2.1. Polymer synthesis

2.1.1. Materials

Methyl trifluoromethansulfonate (MeOTf, initiator, Sigma–Aldrich), was used as received. 2-methyl-2-oxazoline (MOXA, monomer, Sigma–Aldrich) and 4-piperidine ethyl ester (PipEtEst, terminating agent, Sigma–Aldrich) were distilled under reduced pressure. In addition, the former was distilled over KOH as drying agent. Acetonitrile (HPLC grade solvent, Fluka) was freshly destilled after refluxing over CaH_2 under argon atmosphere. N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, Sigma–Aldrich), N-Hydroxysulfosuccinimide sodium salt (sulfo-NHS, Pierce) and 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Fluka) were used as received.

2.1.2. Synthesis of telechelic PMOXA and PLL-g-PMOXA

We have previously reported the synthesis of PLL-g-PMOXA linking the PMOXA-moieties by ester bonds to PLL [33,34]. With the aim of replacing the ester linkage by a tertiary amine functionality to eliminate sensitivity to hydrolysis, the following protocol was developed: Carboxy-terminated poly(2-methyl-2-oxazoline) was synthesized by living cationic ring opening polymerization using MeOTf as initiator and PipEtEst as terminating agent. The degree of polymerization *DP* was adjusted by varying the monomer to initiator ratio. When targeting *DP* = 50, MOXA (8.5 ml, 100 mmol) was dissolved in acetonitrile (30 ml) under argon. MeOTf (219 µl, 2 mmol) was added at 0 °C under argon and the reaction mixture heated to 70 °C for 20 h. Subsequently, the reaction mixture was cooled to room temperature and the reaction was quenched by addition of PipEtEst (925 µl, 6 mmol) and further stirred for 12 h. The solvent was removed to yield a clear colorless oil. After addition of water (MilliQ, 18.3 MOhm cm), the polymers were dialysed in a 1000 MWCO tubing (2 times 24 h; Spectrum Laboratories Inc., Spectra/Por 7) to remove non-polymerized monomers, initiator and terminator and (CH_3)-PMOXA-$(COOC_2H_5)$ was obtained as a white solid after lyophilization. Then, the ester was hydrolyzed by dissolving the polymers in aqueous NaOH of pH 14 and stirring at room temperature for 24 h (CH_3)-PMOXA-(COOH) was similarly dialyzed and lyophilized and obtained as a white powder at an overall yield of approx. 4 g (50%).

Characterization of the bulk polymers was performed by using proton Nuclear Magnetic Resonance (NMR) Spectroscopy (500 MHz, Bruker) and Matrix Assisted Laser Desorption Ionization – Time of Flight (MALDI-ToF) Spectrometry (Bruker Daltonics Ultraflex II).

Fig. 2 shows ^{1}H NMR spectra before (a) and after (b) ester hydrolysis. Peaks originating from the ethyl ester group (B, C in Fig. 2a) are absent after hydrolysis confirming full conversion of ethyl ester into carboxylate groups. Corresponding graphs for PMOXA of higher *DP* are shown in Fig. S1.

Unfortunately it was not possible to independently determine the degree of functionalization *F* at both polymer termini from ^{1}H NMR measurements since the peaks originating from the methyl group at the α-end (A in Fig. 2) and the peaks originating from the piperidine methylene protons at the ω-end (F in Fig. 2) overlap. However, for all polymers Maldi-Tof mass measurements revealed that within the experimental error, the exact expected masses were obtained. Furthermore, very little minor peak distributions were found that mostly correspond to either partial ester hydrolysis that most likely occurred during dialysis ($PMOXA_4$, Fig. 3 and $PMOXA_5$, Fig. S3) or to proton initiation ($PMOXA_8$, Fig. S2). This strongly suggests that the telechelic polymers were obtained in high purity. Furthermore, Maldi-Tof mass spectra show very narrow molecular weight distributions (PDI = 1.01). The degree of polymerization *DP* was independently determined from the mass and ^{1}H NMR spectra. For the latter, the peak integrals originating from the methyl groups of the ethyl ester termini (C in Fig. 2) were related to the peak integrals of the methyl groups of the polymer acetyl groups (E in Fig. 2). With both methods and for all polymers, *DP* was within 15% of the targeted value. Values determined by Maldi-Tof

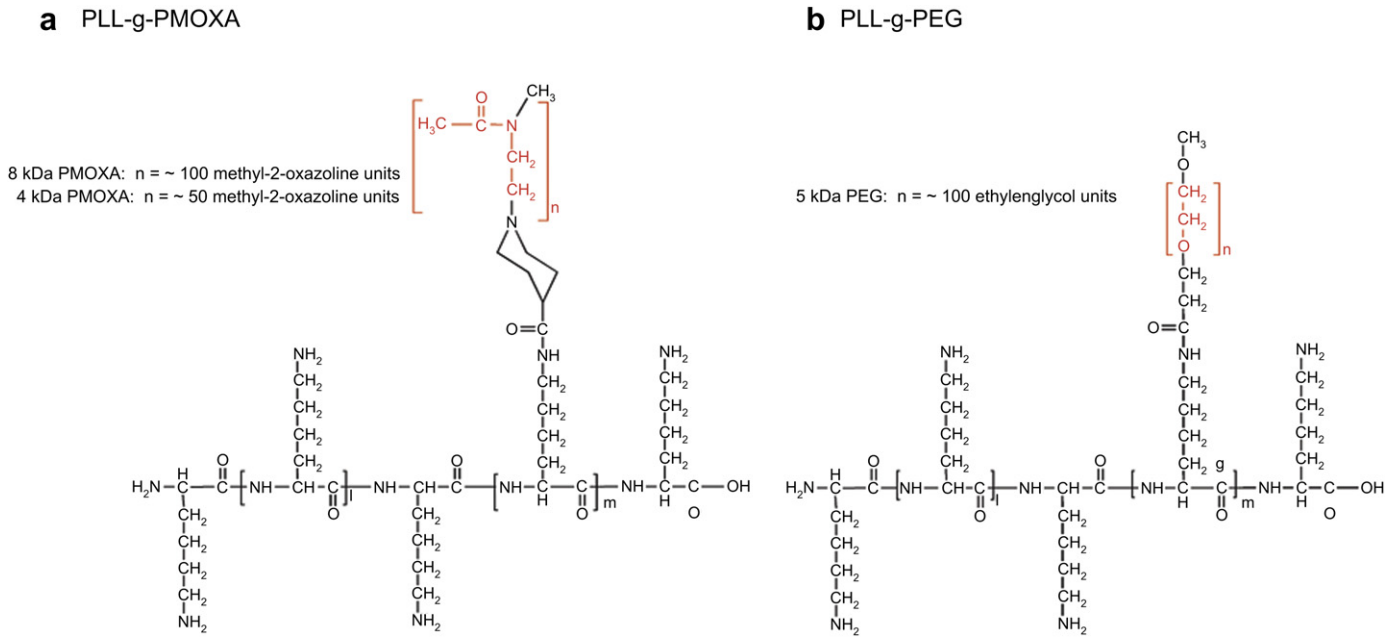

Fig. 1. Comparison of the chemical structure of PLL-*g*-PMOXA- (a) and PLL-*g*-PEG-copolymers (b). Functional PMOXA- and PEG-moieties grafted onto the lysine-groups of the PLL backbone are shown in red. The PLL backbone is shown in its uncharged form. PEG molecular weight of 5 kDa comprises ~100 ethylene glycol units, whereas ~50 and 100 methyl-2-oxazoline units correspond to PMOXA molecular weights of 4 kDa and 8 kDa, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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