



Lysine-based polycation:heparin coacervate for controlled protein delivery



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ABSTRACT

Polycations have good potential as carriers of proteins and genetic material. However, poor control over the release rate and safety issues currently limit their use as delivery vehicles. Here we introduce a new lysine-based polycation, poly(ethylene lysinylaspartate diglyceride) (PELD), which exhibits high cyto-compatibility. PELD self-assembles with the biological polyanion heparin into a coacervate that incorporates proteins with high loading efficiency. Coacervates of varying surface charge were obtained by simple alteration of the PELD:heparin ratio and resulted in diverse release profiles of the model protein bovine serum albumin. Therefore, coacervate charge represents a direct means of control over release rate and duration. The PELD coacervate also rapidly adsorbed onto a porous polymeric scaffold, demonstrating potential use in tissue engineering applications. This coacervate represents a safe and tunable protein delivery system for biomedical applications.

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1. Introduction

Polycations have good potential as carriers of proteins and genetic material. Chitosan and polyethylenimine (PEI) are two polycations commonly used in biomedical applications, including non-viral gene delivery [1–4], vaccine administration [5,6] and delivery of peptides [7,8]. However, safety risks associated with cytotoxicity and the accumulation of nanoparticles following delivery seriously limit their potential [9,10]. Poly(L-lysine) (PLL) has also been widely investigated as a delivery vehicle for proteins and DNA [11–14], though it has similar biocompatibility issues as PEI [15,16]. A synthetic polycation with good biocompatibility and controllable release rate would be highly beneficial for biomedical applications involving protein or gene therapy.

We have previously reported the synthesis of an arginine-based polycation, poly(ethylene arginylaspartate diglyceride) (PEAD), designed to complex with heparin to form a delivery matrix for heparin-binding growth factors [17]. PEAD:heparin self-assembles to form a coacervate, an assortment of organic molecules held together by polyvalent charge attraction and sep-

arated from the aqueous phase. We have demonstrated the use of this coacervate to deliver several factors for stimulating angiogenesis [18], accelerating cutaneous wound-healing [19] and improving heart function post-myocardial infarction [20,21]. In all cases the coacervate showed high loading efficiency, protection from degradation, sustained release over time and enhanced bioactivity compared to the growth factor in free-form. Having demonstrated that the coacervate satisfies the necessary functions of a delivery vehicle, we now address controllability of release rate by modification of the polycation and the coacervate composition.

The polyvalent and ionic nature of the coacervate makes modification of the charge density of the polycation one of the simplest ways to control release rate. Adjusting the polycation:heparin ratio in the coacervate is another simple way. The polycation and growth factor compete for ionic interaction with heparin; thus a coacervate with greater polycation content will compete more, resulting in faster growth factor release, while lower polycation content will slow release. Here we report a new polycation, poly(ethylene lysinylaspartate diglyceride) (PELD), with lysine as the source of positive charge instead of arginine. PEAD has high biocompatibility [17], and here we demonstrate that the same design principle applies to lysine, thus PELD also has excellent biocompatibility. We describe the synthesis and characterization of PELD and its usefulness for controlled release.

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2. Materials and methods

2.1. Materials

Ethylene glycol diglycidyl ether (EGDE) and trifluoroacetic acid (TFA) (TCI America, Portland, OR), t-BOC aspartic acid (BOC-Asp-OH) and t-BOC lysine (BOC-Lys) (Bachem, Torrance, CA), dimethylformamide (DMF), dichloromethane (DCM) and tetra-*n*-butylammonium bromide (TBAB) (Acros Organics, Geel, Belgium), dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) (Alfa Aesar, Ward Hill, MA), 4-dimethylaminopyridine (DMAP) (Avocado Research Chemicals, Lancaster, UK), polyethylenimine (PEI; MW = 50–100 kDa) (MP Biomedicals, Santa Ana, CA), heparin sodium USP (MW = 16 kDa; Scientific Protein Labs, Waunakee, WI), fluorescein (Aldrich Chemical Co, Milwaukee, WI), bovine serum albumin (BSA) (Millipore, Billerica, MA) and poly(D,L-lactide-co-glycolide) 50:50 (PLGA) (Lakeshore Biomaterials, Birmingham, AL) were all used as received.

2.2. Synthesis of PELD

The intermediate, poly(ethylene aspartate diglyceride) (PED), was synthesized as previously described [17]. Briefly, EGDE (1.00 g) was combined with BOC-Asp-OH (1.34 g) and TBAB (5 mg) and dissolved in 0.6 ml of DMF. The mixture was maintained at 120 °C under N₂ for 20 min in a microwave synthesizer (Biotage, Uppsala, Sweden). DMF was evaporated under reduced pressure and TBAB was removed by multiple precipitations in diethyl ether. t-BOC was removed by stirring product in 4:1 DCM:TFA for 2 h. t-BOC lysine was conjugated to PED by DCC/NHS coupling. Briefly, BOC-Lys (0.692 g), NHS (0.323 g), DCC (0.753 g), and DMAP (5 mg) were dissolved in 5 ml DMF. PED (0.864 g) was dissolved in 5 ml of DMF in a separate vial. The two vials were then combined and stirred under N₂ for 24 h. An insoluble dicyclohexylurea by-product formed, and was removed by filtration at 0.22 µm. Finally, t-BOC was removed by stirring in pure TFA for 2 h and the product was purified by multiple precipitations in diethyl ether and then ethyl acetate.

2.3. Characterization of PELD

¹H nuclear magnetic resonance (NMR) spectroscopy was performed with a Biospin Avance NMR spectrometer (Bruker, Billerica, MA) using deuterium oxide (D₂O) solvent. The Fourier transformed infrared (FTIR) spectrum was recorded using a Nicolet IR-100 spectrometer (Thermo, Waltham, MA). Differential scanning calorimetry (DSC) was performed under nitrogen gas at a heating rate of 10 °C min⁻¹ using a Q200 differential scanning calorimeter (TA Instruments, New Castle, DE). The glass transition temperature (*T*_g) was determined using Universal Analysis 2000 software (TA Instruments) as the middle of the glass transition. Gel permeation chromatography (GPC) was performed on a Viscotek VE2001 system equipped with a 270 Dual Detector (differential refractive index and right angle light scattering) (Malvern Instruments, Westborough, MA). Two GRAM columns of 30 and 1000 Å porosities (PSS, Warwick, RI) and dimethylacetamide containing 3 g l⁻¹ lithium bromide and 6 ml l⁻¹ acetic acid were used as the stationary and mobile phases, respectively, and results were compared to polystyrene standards for calibration.

2.4. Preparation of polycation:heparin coacervates

PEAD, PELD and heparin were each dissolved in 0.9% saline and 0.22 µm filter-sterilized. The addition of either polycation to hepa-

rin immediately induced self-assembly of the coacervate, causing the complex to phase separate, visible as a turbid solution.

2.5. Zeta potential and dynamic light scattering (DLS) measurements

PELD and heparin were dissolved in deionized (DI) water at 10 and 1 mg ml⁻¹, respectively, then combined at PELD:heparin mass ratios of 2.5, 5, 7.5, 10, 12.5 and 15. Each coacervate solution was diluted to a 1 ml total volume with DI water. Zeta potential and particle size (hydrodynamic diameter) were both measured using a Zetasizer Nano ZS instrument (Malvern, Westborough, MA). From 10 to 20 readings were taken for each mass ratio and averaged.

2.6. Fluorescent imaging of PELD coacervates

PELD and heparin were dissolved in DI water at 10 mg ml⁻¹ and fluorescein at 10 µg ml⁻¹. A 10 µl volume of fluorescein was first mixed with heparin and then PELD was added to form coacervates with PELD:heparin mass ratios of 5, 10 and 15. The total mass of coacervate (*m*_{PELD} + *m*_{heparin}) was 1 mg for each, prepared in 0.1 ml of water. The coacervates were added to a 96-well plate and imaged immediately using a Nikon Eclipse Ti fluorescent microscope (Nikon Instruments, Melville, NY).

2.7. Cytotoxicity assay

PEAD was synthesized as previously described [17]. NIH 3T3 fibroblasts were seeded at 5 × 10⁴ per well in a 96-well plate and cultured for 12 h at 37 °C/5% CO₂ in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. The medium was then removed and culture medium containing PEAD, PELD or PEI at various concentrations (10, 1, 0.1, 0.01 mg ml⁻¹) was added to four wells per group. After 24 h, cells were washed once with Dulbecco's phosphate-buffered saline and the cell viability was assessed using a Live/Dead Assay (Molecular Probes, Eugene, OR) following the manufacturer's instructions. The results were normalized and compared to a control group which did not receive any polycation added to the media.

2.8. BSA release assay

PELD and heparin were each dissolved in 0.9% saline at 10 mg ml⁻¹. Heparin was initially combined with 500 µg of BSA, then PELD was added to form mass ratios of 6, 8 and 10, corresponding to zeta potentials of approximately -10, -5 and 0 mV, respectively. Tubes were stored at 37 °C under shaking conditions and, after 0, 1, 3, 7, 14 and 21 days, tubes were centrifuged at 12,000g for 5 min and the supernatant removed and frozen at -20 °C. After samples at all the time points had been collected, the BSA concentration was determined using the Pierce 660 nm Protein Assay (Thermo). In order to account for the protein content of the polycations and the heparin in the release fractions, results were normalized to an assay performed identically but without BSA.

2.9. Coacervate adsorption to polymer scaffolds

Porous PLGA scaffolds prepared by the salt-leaching technique were synthesized as previously described [22]. The scaffolds were 1 mm thick and 5 mm in diameter, with a 75–150 µm pore size. PELD and heparin were dissolved in deionized water at 5 mg ml⁻¹ and combined at a 10:1 PELD:heparin mass ratio for maximal turbidity at charge neutrality. Next, 1.2 ml coacervate was added to a glass vial and stirred to prevent settling of the coacervate by gravity. A 50 µl aliquot of coacervate was transferred to a cuvette and

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