Supporting Information

Arginine-Based Biodegradable Ether-Ester Polymers with Low Cytotoxicity as Potential Gene Carriers

Tamar Memanishvili, [†] *Nino Zavradashvili,* [†] *Nino Kupatadze,*[†] *David Tugushi,*[†] *Marekh Gverdtsiteli,*[†] *Vladimir P. Torchilin,**[‡] *Christine Wandrey,*[§] *Lucia Baldi,*[#] *Sagar S. Manoli*[#] *and Ramaz Katsarava**[†]

[†]Institute of Chemistry and Molecular Engineering, Agricultural University of Georgia, University Campus at Digomi, David Aghmashenebeli Alley, 13-th km. 0159, Tbilisi, Georgia

[‡]Center for Pharmaceutical Biotechnology and Nanomedicine, Northeastern University, Boston, MA 02115, USA

[§]Laboratoire de Médecine Régénérative et de Pharmacobiologie and [#] Laboratory of Cellular Biotechnology, École Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland

Corresponding Authors: r.katsarava@agruni.edu.ge, v.torchilin@neu.edu

Materials

L-arginine (Arg), all diols (di-, tri-, and tetra-ethylene glycols), α,ω-alkylene diols (1,2-ethanediol, 1,3-propanediol, 1,6-hexanediol, 1,12-dodecanediol), succinic, adipic, sebacic, and diglycolic acid dichlorides, *N*-hydroxysuccinimide (NHS), *p*-nitrophenol (PNP), dimethylsulfoxide (DMSO), *N,N*-dimethylformamide (DMF), tetrahydrofuran (THF), triethylamine (NEt_3), benzene, isopropanol,

ethyl acetate, acetone, *p*-toluenesulfonic acid monohydrate ($TosOH \cdot H_2O$), and dextrane sulfate (DS) (MW 500 kDa) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without purification. Glycol-*bis*-chloroformates were synthesized according to Ref. [1]

Methods

Characterization of Monomers and Polymers

The chemical structures of the obtained substances were characterized by FTIR and NMR spectroscopy. A Thermo Nicolet Avatar 370 FTIR spectrophotometer coupled with EZ OMNIC software was used for IR analysis. The FTIR spectra of monomers were recorded in fine powders using Avatar Multi-Bounce Flat Plate 45 degree Ge. To obtain the polymer spectra, the samples were crushed between two BaF_2 plates.

The 300 MHz ^{1}H-NMR spectra were recorded on a Varian Mercury 300 spectrometer at 303 K using tetramethylsilane (TMS) as an internal standard and solvents: DMSO-*d*6 for DBC and D_2O for TAAD and cationic polymers.

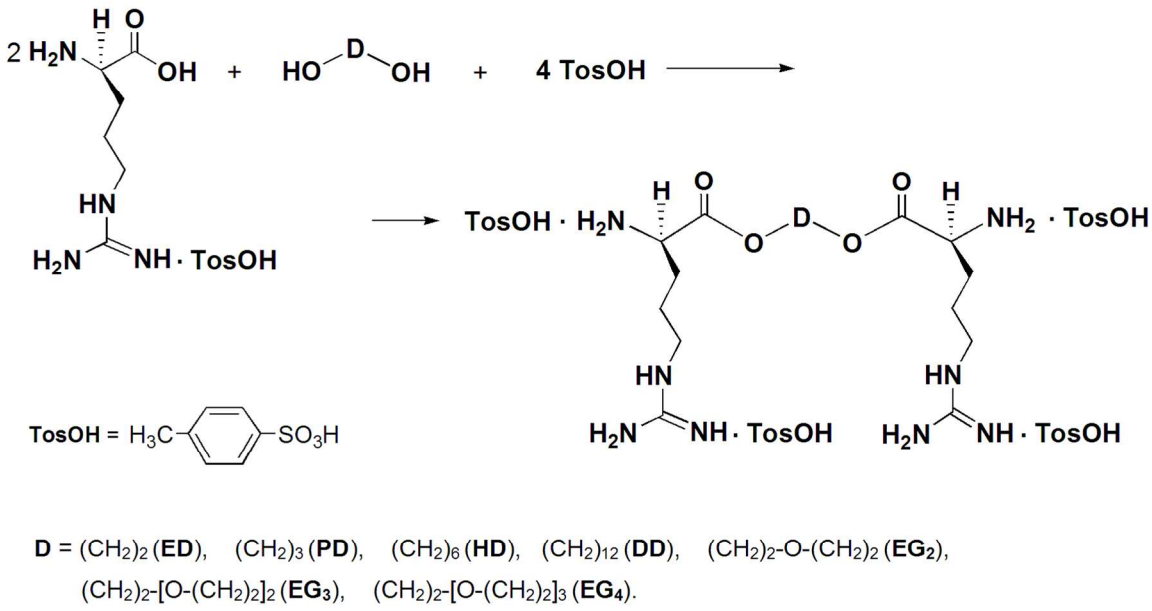
Cellular Uptake

HeLa cells ($0.4x10^6$/well) were seeded in 6-well tissue culture plates, in 2 mL of DMEM medium and cultivated overnight. Meanwhile, the complexes were separately prepared by mixing 1 μg pDNA (pEGFP-N1) with predetermined amounts of polycations (EG_2-Arg-EG_4 or EG_2-Arg-PD) at a weight ratio (WR) 300x and incubating for 30 min at r.t. The cells were incubated with complexes in serum-free medium for 6 h at 37 °C. Naked pDNA was used as the control. Complexes obtained with LT1 transfection reagent (TranslT®-LT1 Mirus Bio Corporation, Madison, WI, USA) were used for comparison. After 6 h of incubation, the medium was removed and the cells were incubated in fresh complete medium for additional 48 h to allow the expression of EGFP protein. The cells

were washed, trypsinized, resuspended in 1 mL cold PBS and then centrifuged at 300 g for 5 min. The cell pellets were rinsed once with cold PBS, resuspended in 500 μL of cold PBS and maintained on ice until the measurement of the cell-associated fluorescence by flow cytometry (Becton–Dickinson FACSort™ flow cytometer, Franklin Lakes, NJ, USA). For each sample 10,000 gated cell counts were analyzed. Cell-associated fluorescence intensities were expressed as the average of these measurements ± S.D. (n = 3).

Synthesis of bis-Nucleophilic Monomers

Key *bis*-nucleophilic monomers - TAAD (Scheme S1) were obtained by direct condensation of amino acid L-arginine with diols in refluxed benzene in the presence of *p*-toluenesulfonic acid monohydrate (TosOH) (at a mole ratio of amino acid/ diol/ TosOH = 2.0/1.0/4.0), as it was reported for the synthesis of *bis*-diamino-diester monomers on the basis of hydrophobic amino acids[2-4]. Seven diols - 1,2-ethanediol (with D=ED), 1,3-propanediol (with D=PD), 1,6-hexanediol (with D=HD), 1,12-dodecanediol (with D=DD), diethylene glycol (with $D=EG_2$), triethylene glycol (with $D=EG_3$) and tetraethylene glycol (with $D=EG_4$) - were used for synthesizing TAAD (For the abbreviations see below).



Scheme S 1. The synthesis of key Arg-based *bis*-nucleophilic monomers TAAD. The labeling of the corresponding TAAD: Arg-ED, Arg-PD, Arg-HD, Arg-DD, Arg-EG_2, Arg-EG_3, Arg-EG_4.

Typically, L-arginine (17.42 g, 0.1 mol), a diol (0.05 mol), *p*-toluenesulfonic acid monohydrate (38.1 g, 0.2 mol) and 270 mL of benzene were placed in a flask equipped with a Dean–Stark apparatus and a stirrer. The heterogenous reaction mixture was refluxed for 24 h until 5.4 mL (0.3 mol) of water evolved. The reaction mixture was then cooled to room temperature (r.t.), benzene was decanted and a new portion of benzene (160 mL) was added to a viscous, tar-like mass obtained and refluxed again for 4 h, cooled to r.t. and left overnight. Benzene was decanted, the product was dried at r.t. in a vacuum and purified by reprecipitation from isopropanol solution into dry ethyl acetate for three times: the dried product was dissolved in 250 mL of hot isopropanol, filtered and 300 mL of dry ethyl acetate was added to the solution, the vessel with the precipitated white sticky mass was stored at 4 °C overnight.

Table S1. Characteristics of key Arg-based *bis*-nucleophilic monomers, TAAD

TAAD*	empirical formula (MM)	yeld, %	m.p. °C	elemantal analysis calculated, % / found, %			
				C	H	N	S
Arg-ED**	$C_{42}H_{62}N_8O_{16}S_4$ (1063.28)	70	66-69	47.44 47.11	5.87 5.91	10.54 10.49	12.06 12.12
Arg-PD[5]	$C_{43}H_{64}N_8O_{16}S_4$ (1077.31)	75	67-71	47.94 47.30	5.98 6.02	10.40 10.34	11.91 11.54
Arg-HD[5]	$C_{46}H_{70}N_8O_{16}S_4$ (1119.39)	71	73-76	49.36 49.01	6.30 6.34	10.01 9.96	11.45 11.51
Arg-DD**	$C_{52}H_{82}N_8O_{16}S_4$ (1203.55)	72	81-84	51.89 51.63	6.87 6.90	9.31 9.26	10.65 10.70
Arg-EG_2**	$C_{44}H_{66}N_8O_{17}S_4$ (1107.33)	63	72-75	47.72 47.28	6.00 6.03	10.12 10.06	11.58 11.64
Arg-EG_3**	$C_{46}H_{70}N_8O_{18}S_4$ (1151.39)	60	71-74	47.98 47.54	6.12 6.16	9.73 9.68	11.14 11.19
Arg-EG_4**	$C_{48}H_{74}N_8O_{19}S_4$ (1195.45)	65	61-64	48.23 47.68	6.24 6.60	9.37 9.02	10.31 10.37

*For designations of TAAD see Scheme S1.

** Synthesized for the first time. Arg-PD and Arg-HD were reported in[5].

Next day, the mixture of solvents was decanted and the reprecipitation procedure was repeated twice. The purification process was monitored by thin-layer chromatography (TLC) using ALUGRAM sheets SIL G/UV254 (Macherey-Nagel, Germany) and a solvent mixture of benzene/ethanol (3:2). According to TLC, the products were free of admixtures after the third reprecipitation. The products were dried in a vacuum at 50 °C up to constant weight. White hygroscopic powders were obtained. The basic characteristics (yields, melting points, results of elemental analysis) of *bis*-nucleophilic monomers are summarized in Table S1. Two Arg-based monomers - Arg-PD and Arg-HD were reported in[5] although no m.p and data of elemental analysis were given. Therefore, we cite these data in Table S1 as well.

The ^{1}H NMR data of TAAD obtained for the first time are given below (chemical shifts δ against TMS in ppm, coupling constant J in Hz):

Arg-ED: 1.76-2.03 (8H, m, CH$\underline{CH_2CH_2}$); 2.46 (12H, s, $\underline{CH_3}$-C_6H_4); 3.26 (4H, t, *J*=6.7, NCH_2); 4.23 (2H, t, *J*=6.4, CH); 4.43 (4H, t, *J*=6.4, CH_2OC=O); 7.38 -7.43 (8H, m, $HOSO_2$-$\underline{H_2}C_6H_2$-CH_3) & 7.71-7.76 (8H, m, $HOSO_2$-$H_2C_6\underline{H_2}$-CH_3).

Arg-DD: 1.23-2.10 (28H, m, CH$\underline{CH_2CH_2}$ & $CH_2\underline{CH_2CH_2}$); 2.42 (12H, s, CH_3); 3.21 (4H, t, *J*=6.9, NCH_2); 4.16 (2H, t, *J*=6.3, CH); 4.17 - 4.32 (4H, m, CH_2OC=O); 7.35-7.41 (8H, m, $HOSO_2$-$\underline{H_2}C_6H_2$-CH_3) & 7.72-7.78 (8H, m, $HOSO_2$-$H_2C_6\underline{H_2}$-CH_3).

Arg-EG_2: 1.74-2.03 (8H, m, CH$\underline{CH_2CH_2}$); 2.44 (12H, s, $\underline{CH_3}$-C_6H_4); 3.19 (2H, t, *J*=6.8) & 3.23 (2H, t, *J*=6.8, NCH_2); 3.85 (4H, m, CH_2OCH_2); 4.21 (2H, t, *J*=6.4, CH); 4.45 (4H, m, CH_2OC=O); 7.35-7.41 (8H, m, $HOSO_2$-$\underline{H_2}C_6H_2$-CH_3) & 7.70-7.75 (8H, m, $HOSO_2$-$H_2C_6\underline{H_2}$-CH_3).

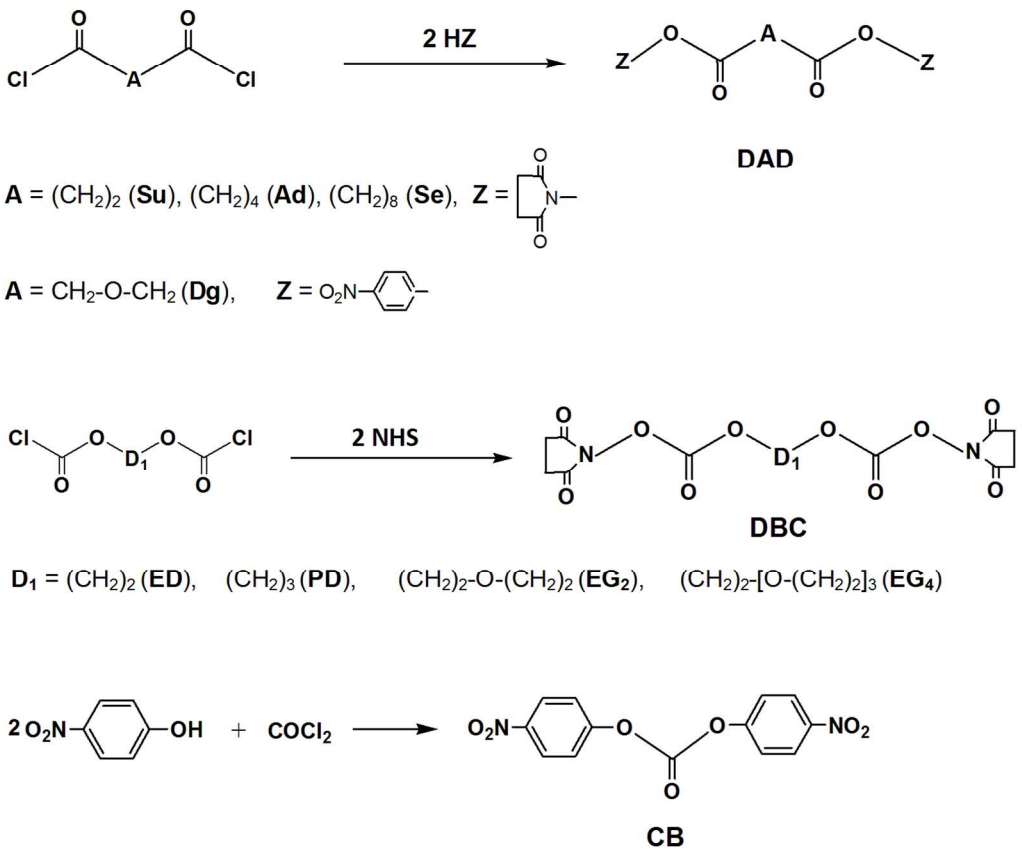
Arg-EG_3: 1.63-2.14 (8H, m, CH$\underline{CH_2CH_2}$); 2.44 (12H, s, $\underline{CH_3}$-C_6H_4); 3.22 (4H, t, *J*=6.9, NCH_2); 3.72-3.87 (8H, m, CH_2OCH_2); 4.22 (2H, t, *J*=6.5, CH); 4.44 - 4.48 (4H, m, CH_2OC=O); 7.39-7.44 (8H, m, $HOSO_2$-$\underline{H_2}C_6H_2$-CH_3) & 7.72-7.77 (8H, m, $HOSO_2$-$\underline{H_2}C_6H_2$-CH_3).

Arg-EG_4: 1.65-2.15 (8H, m, CH$\underline{CH_2CH_2}$); 2.46 (12H, s, $\underline{CH_3}$-C_6H_4); 3.26 (4H, t, *J*=6.7, NCH_2); 3.68-3.86 (12H, m, CH_2OCH_2); 4.24 (2H, t, *J*=6.3, CH); 4.48 (4H, m, CH_2OC=O); 7.38-7.43 (8H, m, $HOSO_2$-$\underline{H_2}C_6H_2$-CH_3) & 7.71-7.76 (8H, m, $HOSO_2$-$\underline{H_2}C_6H_2$-CH_3).

In the FTIR spectra of the new TAAD all the basic absorption bands were observed (cm-1): 1720-1740 [-C(O)-O-], 1120 (-CH_2-O-CH_2- in EG_2, EG_3, and EG_4 based monomers), 3000-3360, broad [-NH_2.HOTos & –NHC(=NH)NH_2.HOTos].

Synthesis of bis-Electrophilic Monomers

Three classes of *bis*-electrophilic monomers (Scheme S2): (*i*) dicarboxylic acid diesters (DAD), (*ii*) diol *bis*-carbonates (DBC) and (*iii*) carbonate (CB), were obtained for synthesizing three classes of the goal Arg-based polymers. (i) DAD on the basis of succinic (with Su) and adipic (with Ad) acids were synthesized by interaction of the corresponding dichlorides (1 mol) with NHS (2 mol) as reported previously[6]. DAD on the basis of sebacic acid (with Se) was synthesized *via* sebacoyl chloride using the same procedure. DAD on the basis of diglycolic (with Dg) acid was obtained by interaction of the corresponding dichloride (1.0 mol) with PNP (2.0 mol) as reported in[7.] The synthesis of DAD *via* diacid chlorides is represented in Scheme S 2; (ii) DBC were obtained by interaction of the corresponding glycol-*bis*-chloroformates (1.0 mol) with NHS (2.0 mol) by continuously stirring in 250 mL of dry acetone (or tetrahydrofurane) in the presence of pyridine as HCl acceptor. *Bis*-cloroformates on the basis of four diols - 1,2-etanediol (with D_1=ED), 1,3-propandiol (with D_1=PD), diethylene glycol (with D_1=EG_2) and tetraethylene glycol (with D_1=EG_4) used for synthesizing DBC, were obtained by interaction of corresponding diols with phosgene according to Ref. [1]. The synthesis of DBC *via* glycol-*bis*-chloroformates is represented in Scheme S2. All the DBC used in the present work were synthesized for the first time; (iii) CB was synthesized by interaction of phosgene with PNP (Scheme S2) as described in[8]. The yields, solvents for recrystallization, melting points, and data of elemental analysis of *bis*-electrophilic monomers are summarized in Table S2. For all the abbreviations see below.



Scheme S 2. The synthesis of activated *bis*-electrophilic monomers: diacid diesters (DAD), glycols *bis*-carbonates (DBC), and carbonate (CB).

The ^{1}H NMR data of DBC obtained for the first time (Table S2) are given below (chemical shifts δ against TMS in ppm, coupling constant J in Hz):

DBC, D_1=ED: 2.80 (8H, s, CH_2C=O), 4.68 (4H, s, CH_2OC=O)

DBC, D_1=PD: 2.24 (2H, m, J=6.3, $OCH_2\underline{CH_2}CH_2O$), 2.80 (8H, s, CH_2C=O), 4.48 (4H, t, J=6.3, CH_2OC=O).

DBC, D_1=EG_2: 2.80 (8H, s, CH_2C=O); 3.81 (4H, m, CH_2OCH_2); 4.49 (4H, m, CH_2OC=O).

DBC, D_1=EG_4: 2.80 (8H, s, CH_2C=O); 3.66-3.83 (12H, m, CH_2OCH_2); 4.51 (4H, m, CH_2OC=O).

Table S 2. The basic properties of key *bis*-electrophilic monomers: DAD, DBC, and CB

monomers	empirical formula (MM)	yeld, %	m.p. °C (Lit. m.p)	solvent for recrystalization	elemantal analisis calculated, % found, %		
					C	H	N
DAD A= HZ=							
Su NHS		60	296-297 (295-297)[6]	1,4-Dioxane			
Ad NHS		58	171-173 (172-174)[6]	1,4-Dioxane			
Se NHS		70	167-169 (162-167)[9]	Acetone			
Dg PNP		81	167-168 (166-167)[7]	Toluene			
DBC, D_1							
ED*	$C_{12}H_{12}N_2O_{10}$ (344.24)	62	182-185	Acetone/Water 2/1	41.87 41.66	3.51 3.53	8.14 8.09
PD*	$C_{13}H_{14}N_2O_{10}$ (358.27)	60	117-119	Ethyl acetate	43.58 43.36	3.94 3.96	7.82 7.78
EG_2*	$C_{14}H_{16}N_2O_{11}$ (388.29)	65	130-131	Toluene	43.31 43.29	4.15 4.12	7.22 7.18
EG_4*	$C_{14}H_{24}N_2O_{13}$ (476.38)	58	86-88	Toluene/CH_2CL_2 2/1	45.38 45.15	5.07 5.10	5.88 5.85
CB		85	142-143 (141-142)[8]	Benzene			

* Synthesized for the first time.

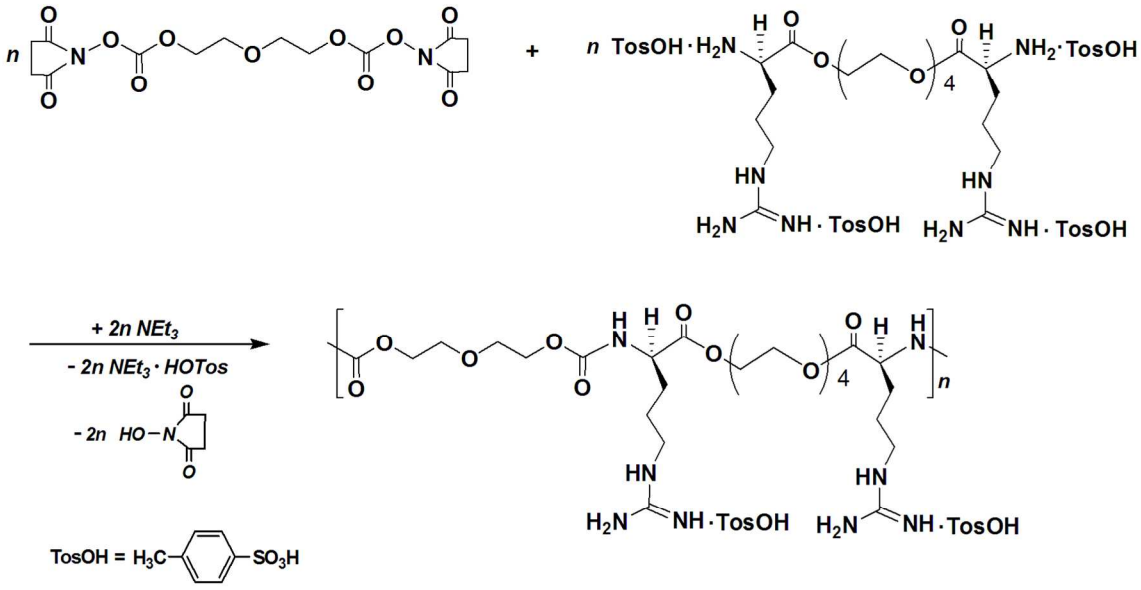
In the FTIR spectra of all the given DBC, the basic bands were observed (cm^{-1}):

1730, 1780, 1810 broad [-C(O)-O- and imide cycle], 1380 (-N=), 1130, 1210 ($-CH_2-O-CH_2-$) .

Polymer Synthesis

The goal polymers (for the structures see Scheme 1) were synthesized by polycondensation of Arg-based key monomers, (TAAD, 1.0 mol), with activated diesters of different classes (DAD, DBC, or CB, 1.0 mol) using TEA (slight excess, 2.2 mol) as a *p*-toluolsifonic acid acceptor. The reactions were carried out in DMSO similar to the synthesis of biodegradable polymers composed of hydrophobic-amino acids[2-4].

Here, the synthesis of PEEUR EG_2-Arg-EG_4 (Scheme S3) is given as a typical example to illustrate the principle of the polymer synthesis. A mixture of tetra-*p*-toluenesulfonic acid salt of 11.95g (0.01 mol) of *bis*-(ʟ-arginine)-3,6,9-trioxa-1,11-undecylene diester (TAAD from Scheme S1, D = EG_4) and 3.88 g (0.01 mol) *bis*-succinimidyl-3-oxa-1,5-pentylene-dicarbonate (DBC from Scheme S2, D_1 = EG_2) was dissolved in 5.25 mL of dry DMSO at r.t. on stirring and 3.08 mL (0.022 mol) of TEA was added (solution concentration 1.2 mol/L). The reaction mixture was stirred at 50-60 °C for 24 h. The obtained viscous solution was cooled to r.t., diluted with 8 mL of DMSO and poured drop-wise into dried (using molecular sieves 4 A) acetone where the polymer precipitated as gum-like sticky mass. The liquid phase (DMSO + acetone) was decanted, the polymer obtained was washed for several times with fresh portions of chilled acetone, then 100 mL of dry acetone was added and refluxed for 2-3 h to remove low-molecular-weight fractions and impurities. The polymer was dried at 40-50 °C in a vacuum up to constant weight. All other polymers (Table 1) were synthesized, purified and dried analogously. All polymers were hygroscopic.



Scheme S 3. The synthesis of Arg-based PEEUR EG_2-Arg-EG_4

The ^{1}H NMR data of the four polymers used in biological experiments (chemical shifts δ against TMS in ppm):

EG_2-Arg-PD: 1.57-1.91 (8H, m $CH\underline{CH_2CH_2}$); 2.09 (2H, m, $OCH_2\underline{CH_2}CH_2O$); 2.45 (6H, s, $\underline{CH_3}$-C_6H_4); 3.25 (4H, br., NCH_2); 3.80 (4H, m, CH_2OCH_2); 4.26 (2H, br., CH); 4.27-4.41 (8H, m, $CH_2OC{=}O$); 7.42 (4H, m, $HOSO_2$-$\underline{H_2}C_6H_2$-CH_3) & 7.76 (4H, m, $HOSO_2$-$\underline{H_2}C_6H_2$-CH_3).

EG_2-Arg-EG_4: 1.60-1.95 (8H, m, $CH\underline{CH_2CH_2}$); 2.45 (6H, s, $\underline{CH_3}$-C_6H_4); 3.23(4H, br., NCH_2); 3.73-3.82(16H, m, CH_2OCH_2); 4.20 (2H, br.,CH); 4.27-4.38 (8H, m, $CH_2OC{=}O$); 7.42 (4H, m, $HOSO_2$-$\underline{H_2}C_6H_2$-CH_3) & 7.75(4H, m, $HOSO_2$-$\underline{H_2}C_6H_2$-CH_3)

CO-Arg-ED: 1.54 - 2.02 (8H, m, CH$\underline{CH_2CH_2}$); 2.46 (6H, s, $\underline{CH_3}$-C_6H_4); 3.21 (4H, m, NCH_2); 4.30 (2H, m, CH); 4.46 (4H, m, $CH_2OC{=}O$); 7.42 (4H, m, $HOSO_2$-$\underline{H_2}C_6H_2$-CH_3) & 7.76 (4H, m, $HOSO_2$-$\underline{H_2}C_6H_2$-CH_3).

CO-Arg-PD: 1.53 - 2.00 (8H, m, CH$\underline{CH_2CH_2}$); 2.10 (2H, m, $OCH_2\underline{CH_2}CH_2O$); 2.46 (6H, s, $\underline{CH_3}$-C_6H_4); 3.20 (4H, m, NCH_2); 4.26 (2H, m, CH); 4.40 (4H, m, $CH_2OC{=}O$); 7.42 (4H, m, $HOSO_2$-$\underline{H_2}C_6H_2$-CH_3) & 7.76 (4H, m, $HOSO_2$-$\underline{H_2}C_6H_2$-CH_3).

The FTIR data of the four polymers used in biological experiments:

EG_2-Arg-PD: (cm^{-1}): 1650-1750 [-C(O)-O- & -NH-C(O)-O-], 1100- (-CH_2-O-CH_2-), 3000-3500 [-NH-C(O)- & NHC(=NH)NH_2.HOTos].

EG_2-Arg-EG_4 (cm^{-1}): 1650-1750 [-C(O)-O- & -NH-C(O)-O-], 1100- (-CH_2-O-CH_2-), 3000-3500 [-NH-C(O)- & NHC(=NH)NH_2.HOTos].

CO-Arg-ED (cm^{-1}): 1650-1735 [-C(O)-O- & -NH-C(O)-O-], 1119 (-CH_2-O-CH_2-), 3000-3363 [-NH-C(O)- & NHC(=NH)NH_2.HOTos].

CO-Arg-PD (cm^{-1}): 1650-1735 [-C(O)-O- & -NH-C(O)-O-], 1119 (-CH_2-O-CH_2-), 3000-3363 [-NH-C(O)- & NHC(=NH)NH_2.HOTos].

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