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Solution Properties and Potential Biological Applications of Zwitterionic Poly(ε-*N*-methacryloyl-L-lysine)

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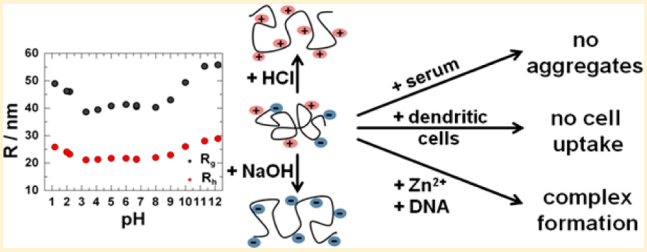
Supporting Information

ABSTRACT: Poly(ε-*N*-methacryloyl-L-lysine) (PMALys) was synthesized by free radical polymerization yielding a zwitterionic polymer with M_w = 721 000 g mol^{-1}. The polymer dissolves in pure water as well as in aqueous salt solution up to 5 M $NaClO_4$ and over wide range of pH values (1.3 ≤ pH ≤ 12.7) as single chains without any sign for aggregate formation. The zwitterionic polymer shows an expanded random coil structure at and close to isoelectric conditions and further expands upon addition of acid and base, respectively. The polymer fulfills four major prerequisites for a promising nano carrier in potential biomedical applications: (1) It is biocompatible, indicated by a low cytotoxicity. (2) It does not aggregate in concentrated human blood serum solution. (3) The amino groups in the polyzwitterion may be utilized for conjugations as demonstrated by labeling reactions with AlexaFluor488. (4) Cell uptake experiments revealed little uptake in bone marrow dendritic cells, i.e., little *unspecific* uptake, which is mandatory for a successful specific targeting of cells. Finally, upon addition of Zn^{2+} ions the polyzwitterions may be converted into polycations which are demonstrated to form complexes with DNA. Such complexes may be advantageous for application in gene transfection studies.



INTRODUCTION

Polyampholytes and zwitterionic polymers represent an interesting and frequently investigated class of ionic polymers[1−20] which change their total charge as a function of pH. The vast majority of zwitterionic polymers comprise betanoic side chains such as sulfo-, carboxy-, and phosphobetaines. Compared to neutral polymers, the characterization of zwitterionic polymers is mostly restricted to simple measurements of the intrinsic viscosity, which usually exhibits a minimum around the IEP. In addition, most of the zwitterionic polymers are insoluble or poorly soluble in pure water but become expanded at high added salt concentration. Quantitative characterization in terms of absolute molar mass and chain dimension such as the radius of gyration, R_g, and the hydrodynamic radius, R_h, are rare.[21−30]

Although polymers with various amino acids in the side chains are frequently reported,[31−41] zwitterionic polymers incorporating amino acids in the side chains[42−46] have not received much attention although such polymers may be expected to show a good biocompatibility and low protein adsorption[45−47] as known for other polybetaines.[48−57] Long ago, Morawetz and Sammak[42] have described the synthesis of poly(ε-*N*-methacryloyl-L-lysine) (PMALys) and investigated the Cu^{2+} chelate formation. Later, chelates with other transition metal ions such as Ni^{2+} and Zn^{2+} were investigated.[43,44]

The present work reports on the conformational properties of poly(ε-*N*-methacryloyl-L-lysine) as a function of added salt and pH and investigates the suitability as multifunctional "nanocarriers" in biological applications. The subject of polymeric nanocarriers is a frequently addressed topic within the advancing field of "nanomedicines". In particular, for "in vivo" applications we have defined four fundamental properties nanocarriers should fulfill prior to more specific biomedical applications: (1) The nanocarrier must be biocompatible, indicated by a sufficiently low cytotoxicity. (2) The nanocarrier must not aggregate even in concentrated human blood serum solution. (3) The nanocarrier should be multifunctional and allow for versatile chemical conjugation reactions. (4) If

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nanoparticles are to be utilized for specific targeting of cells, they must not show any unspecific uptake. Whereas point 1 and partly point 3 are usually considered in the literature, points 2 and 4 are frequently ignored, until eventually the anticipated biomedical goal could not be reached. Thus, a major intention of the present work is to demonstrate the feasibility of profound suitability tests on one particular polyzwitterion that may turn out to become a promising polymeric nanocarrier.

EXPERIMENTAL SECTION

Synthesis of ε-*N*-Methacryloyl-L-lysine (6). The ε-*N*-methacryloyl-L-lysine copper complex (**2**) was synthesized according to the procedure described by Nagaoka et al.[58]

The noncomplexed monomer ε-*N*-methacryloyl-L-lysine (**6**) was obtained by precipitation of the less soluble copper oxalate (K_{sp} = 4.43 × 10^{-10} mol L^{-1}) and Chelex 100 complex with subsequent column purification. In a typical experiment the ε-*N*-methacryloyl-L-lysine–copper complex (17.8 g, 360 mmol) was suspended in 1 N NaOH solution (285 mL). To the blue suspension sodium oxalate (**4**) (9.79 g, 720 mmol) in water (285 mL) was added and kept stirring for 3 days. After centrifugation the light blue supernatant was separated from the brown precipitate. For complete removal of the copper (~5 ppm) Chelex 100 (**5**) (28.5 g) was added to the supernatant, and the suspension was stirred for 1 h. After filtration the colorless solution (pH 12) was freeze-dried (17.8 g). This procedure is known to reduce the copper concentration to less than 0.1 ppb. To remove the residual NaOH salt, ion-exchange column chromatography was performed. The column was loaded with a suspension of ion-exchange resin Ag 1 Resin chloride form from Biorad, (125 g) in water (300 mL) and converted into Ag 1 Resin hydroxide form according to LeMaster et al.[59] The column was loaded with ε-*N*-methacryloyl-L-lysine (17.8 g in 150 mL of water) and washed with water and 1% formic acid. The product eluted at pH 7–8. The solution was filtered (VV 0.1 μm) and freeze-dried. Yield: 10.5 g (67%).

The ^{1}H NMR spectrum and HPLC elution curve for ε-*N*-methacryloyl-L-lysine are shown in the Supporting Information, Figures S1 and S2.

Synthesis of Poly(ε-*N*-methacryloyl-L-lysine) (8). In a typical polymerization the initiator 4,4-azobis-4-cyano valeric acid (**7**) (2.5 mg, 8.9 × 10^{-3} mmol = 0.4 mol % with respect to monomer), dissolved in water (8 mL), was added to the monomer (**6**) (0.5 g, 2.333 mmol). The solution (pH = 6.7) was degassed by three freeze–pump cycles and subsequently polymerized at 65 °C for 9 days. Then the reaction mixture was diluted with water (15 mL), and the residual monomer was removed by dialysis (Amicon Centrifuge Tubes, 3K). After freeze-drying a colorless solid was obtained. Yield: 50% (0.25 g) after dialysis. Poly(ε-*N*-methacryloyl-L-lysine) was soluble in water and characterized by static (see Figure 1) and dynamic (see Supporting Information Figure S3) light scattering.

Labeling of Poly(ε-*N*-methacryloyl-L-lysine) with Alexa Fluor 488. To a solution of poly(ε-*N*-methacryloyl-L-lysine) (31 mg, 4.3 × 10^{-8} mol) in 1x PBS (3.1 mL), a solution of Alexa Fluor 488 TFP Ester (1:7.4) (1.4 mg) in DMSO (0.2 mL) was added. The reaction mixture was incubated at 20 °C overnight and purified with Amicon Ultra Centrifugal Filter Devices 3 kDa at 4000*g*, 15 °C for 20 min approximately 10 times until the filtrate was colorless.

The quantum yield loss of the conjugated as compared to the free dye is negligible as determined by fluorescence spectroscopy.

GPC. The GPC consisted of a VWR 7614 degasser, a Hitashi L-2130 pump, a Hitachi L2350 column oven, a L2490 RI detector, and 3 PSS Novema columns (8.0 × 300 mm) 30, 300, 3000 with 10 μm particle size. Measurements were conducted at 25 °C at a flow rate of 1 mL/min using aqueous 0.1 M $NaNO_3$ solution as eluent. The samples were dissolved in the eluent with a concentration of c = 3 g L^{-1}, and 20 μL of the sample was injected. For the determination of the molecular weight Pullulan standards with molecular weight range from 5900 to 788 000 g mol^{-1} were used.

Static and Dynamic Light Scattering. Static light scattering (SLS) measurements were performed with an ALV-SP86 goniometer, an ALV-3000 correlator, a Uniphase HeNe laser (25 mW output power at λ = 632.8 nm wavelength), and an ALV/High QE APD avalanche diode fiber-optic detection system. An ALV-SP125 goniometer, an ALV-5000 correlator, and a Spectra-Physics 2060 argon ion laser (500 mW output power at λ = 514.5 nm wavelength) were utilized for dynamic light scattering (DLS) measurements. The scattered intensity was divided by a beam splitter (approximately 50:50), each portion of which was detected by a photomultiplier. The two signals were cross-correlated in order to minimize nonrandom electronic noise.

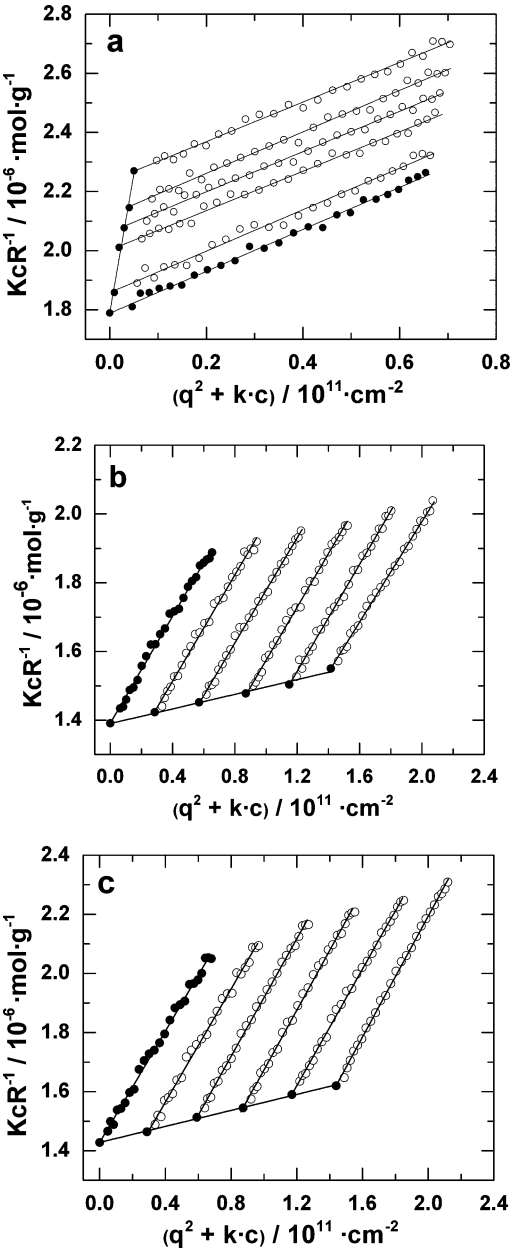

Figure 1. Zimm plots for poly(ε-*N*-methacryloyl-L-lysine) in water (a), 0.1 M $NaClO_4$ (b), and 5 M $NaClO_4$ (c) solution.

Solutions were typically measured from 30° to 150° in steps of 5° (SLS) or in steps of 15° or 20° (DLS). The static scattering intensities were analyzed according to Zimm in order to yield the weight-average molar mass, M_w, the square root of z-average mean square radius of gyration, $R_g = \langle R_g^2 \rangle_z$, and the second viral coefficient A_2. The experimental uncertainties are estimated to be ±5% for M_w and R_g. The correlation functions showed a monomodal decay and were fitted by a sum of two exponentials, from which the first cumulant Γ was calculated. The z-average diffusion coefficient D_z was obtained by

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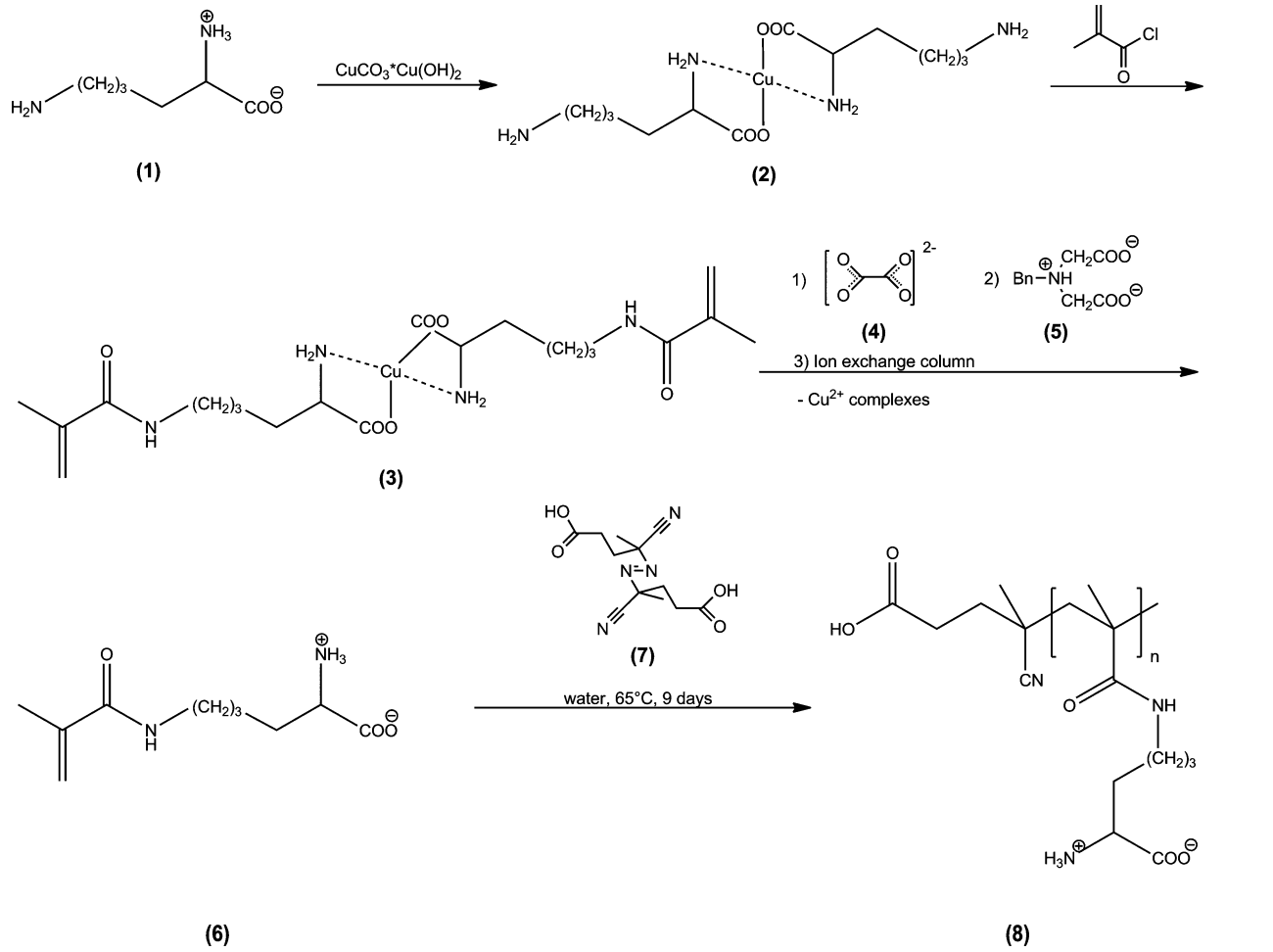
Scheme 1. Synthesis of Poly(ε-*N*-methacryloyl-L-lysine)



extrapolation of Γ/q^2 for to $q = 0$, leading to the inverse z-average hydrodynamic radius $R_h = \langle 1/R_h \rangle_z^{-1}$ by formal application of Stokes' law. The experimental uncertainties are estimated to ±2% for R_h.

Stock solutions of each sample were prepared at $c = 1$ g L^{-1} and filtered through 0.2 μm pore size Millipore GS filters into 20 mm diameter quartz cuvettes (Hellma). Further dilutions were made by subsequent addition of 0.1 μm pore size Millipore VV filtered solvent into the LS cuvette, and the respective concentrations were obtained by weighing.

The refractive index increments at $\lambda = 632.8$ nm wavelength were measured by a home-built Michelson interferometer as described elsewhere.[60] The values for dn/dc were determined to be 0.1904×10^{-3} and 0.1651×10^{-3} g dm^{-3} for the polymer in 0.1 and 5 M $NaClO_4$. The experimental uncertainties are estimated to ±2% for dn/dc.

For the DLS measurements of the polyzwitterions in human serum the following procedure was applied: An equal volume of the polyzwitterion solution in PBS-buffer ($c = 1$ g/L) was filtered to undiluted human serum sample leading to the final polymer concentration $c = 0.5$ g/L in a 50% diluted serum with an approximate total protein concentration of >30%. The cuvette was gently shaken for 20 min after addition of the polyzwitterions, and the angular dependent DLS measurements take typically 1 h. No change in the aggregation behavior was observed during the measurements.

Fluorescence Correlation Spectroscopy (FCS). The experiments were conducted on a commercial setup combining an inverted microscope IX70, a FluoView300 confocal laser scanning unit (both Olympus), and a FCS unit (PicoQuant) consisting of a single-photon avalanche diode (τ-SPAD) and the time-correlated single-photon counting card TimeHarp 200. Excitation was done by an argon ion laser at $\lambda = 488$ nm (8 mW, CVI Melles Griot), and the fluorescence signal was detected after filtering with a LP488R Raman edge long-pass filter. A water immersion objective Olympus UPLSAPO 60XW was used for the measurements. Further details on the FCS experiments and data evaluation are given in the Supporting Information.

Zeta-Potential. Zeta-potential was measured using a Zetasizer Nano ZS from Malvern Instruments and disposable folded capillary cells. Samples were prepared with a concentration of $c = 0.2$ g L^{-1} in aqueous 5 mM citric acid–Na_2HPO_4 buffer solutions with pH ranging from 2.8 to 8.6. The solutions were filtered through 0.2 μm pore size Millipore GS filters and degassed.

Isoelectric Focusing. Determination of the isoelectric point of the dye-labeled polyzwitterion was accomplished via isoelectric focusing using (a) a BIO-RAD "Micro-Rotaphor Preparative IEF-Cell" and (b) IPG strips.

For (a) 200 μL of salt-free aqueous polymer solution with $c = 1$ g L^{-1} was mixed with 2740 μL of water and 60 μL of Fluka Ampholyte solution (pH 3–6). The focusing chamber was loaded with the polymer–ampholyte solution, and the polymer was focused for 4 h at 20 °C with 1–200 V and 1 W. After 4 h the samples were removed from the 10 chambers, and pH of each chamber was measured. The polymer concentration of each chamber was determined by UV measurements on a Cary 100 Bio UV–vis spectrometer (Varian, Inc.) and FCS measurements.

For (b) 160 μg of AF488-labeled polymer was diluted in 500 μL of rehydration buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v) carrier ampholytes (SERVALYT 3-10, SERVA electrophoresis), and 0.001% (w/v) Bromophenol blue. The sample was loaded onto an immobilized pH gradient (IPG) strip (SERVA

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IPG BlueStrip 3-10/24 cm, SERVA Electrophoresis) by overnight passive in-gel rehydration at 20 °C. Rehydrated strips were focused using an IEF100 isoelectric focusing unit (Hoefer Inc.) at 20 °C under a layer of Immobiline DryStrip Cover Fluid (GE Healthcare). For improved sample entry, initial voltage was limited to 500 V for 1 h and then increased with a gradient up to 8000 V using a total of 75 000 V/h. After IEF, the strip was incubated with 10% (v/v) acetic acid and 35% (v/v) methanol for 2 h, before it was imaged using a LAS-3000 Imager (Fujifilm).

Cell Culture. Bone marrow was extracted from femurs and tibiae of C57BL/6 mice and filtered through filters of 40 μm pore size, in order to produce a single cell dispersion. The number of cells was determined microscopically utilizing a Neubauer counting unit and adjusted to 3×10^6 cells in 4 mL of RPMI-Medium plus GM-CSF (4 ng/mL). The cells were placed on six-well plates for 6 days in an incubator with 5% CO_2. After 3 days 4 mL of fresh medium was added to each of the wells.

CLSM. Cells were stained with 1 μg/mL Hoechst 33342 solution and with 2.5 μg/mL Cellmask Orange (Invitrogen, Germany) solution directly before imaging. The cells were imaged in Nunc Lab-Tek Chambered Coverglasses using a Zeiss LSM 710 NLO confocal laser scanning microscope (Carl Zeiss Microimaging GmbH, Jena, Germany), using 405 nm (Hoechst 33342), 488 nm (Alexa Fluor 488), and 543 nm (CellMask orange) laser lines. The image analysis was performed using the Zeiss Zen-2009 software (Carl Zeiss Microimaging GmbH, Jena, Germany).

FACS. Measurements were performed on a LSR II flow cytometer from BD Bioscience equipped with a laser with excitation wavelengths at 488, 633, 355, and 405 nm. 1×10^{12} particles and 500 000 cells were analyzed per well. DiVa Digital Software was used to operate the instrument and to evaluate the data.

Toxicity Tests. BM-DCs (bone marrow-derived dendritic cells) were resuspended in cell culture media containing polymers at concentrations indicated and were seeded into wells of a 96-well cell culture plate in triplicates (each 2.5×10^5 cells/100 μL). One day later, viability was assessed by MTT assay. Data represent mean ± SEM, normalized in each experiment to the viability of untreated BM-DCs. Data are compiled from two independent experiments.

HEK293 cells (each 2×10^4) were seeded into wells of a 96-well cell culture plate in triplicates. One day later, cell culture media was replenished with media containing polymer at concentrations indicated (each 100 μL). After another day, viability was assessed by MTT assay. Data represent mean ± SEM, normalized in each experiment to the viability of HEK293 cells left untrated. Data are compiled from two independent experiments.

Complexation Experiments. An aqueous solution of polyzwitterion (c = 0.4 g L^{-1}) was filtered through Millex GS filters (220 nm pore size) into dust-free Suprasil cuvettes. A predetermined amount of metal ion solution was filtered (Millex GV, 100 nm pore size) directly into the light scattering cuvettes in a dust-free flow cabinet, such that the final polyzwitterion concentration was 50 mg L^{-1} and the Me^{2+}/COO^- ratios as given in the text. Subsequently, a solution of pUC19 plasmid DNA (2682 bp, c = 50 mg L^{-1}) was filtered through Millex GHP filters (200 nm pore size) into the light scattering cuvette. After each addition the cuvettes were closed by a dust-free Teflon stopper and gently shaken. The Me^{2+}/PO_4^- ratio was determined by weighing.

RESULTS AND DISCUSSION

Synthesis and Characterization. Poly(ε-*N*-methacryloyl-L-lysine) was synthesized according to Scheme 1. Reliable GPC measurements were difficult to obtain because pronounced interactions with the column material resulted in an anomalous elution behavior. Eventually successful measurements could be achieved utilizing the setup described above, and the results are shown in Table 1.

The elution curves do show some tailing, which is caused either by a small molar mass fraction or by an anomalously eluting larger molar mass fraction. Therefore, the results of two data evaluation procedures are included in Table 1. If tailing is ignored, the PDI is close to the expected Schulz−Flory molar mass distribution; if tailing is included, a broader PDI = 2.8 results. Since the difference is not too large, we refrained from further fractionation.

Table 1. GPC Molar Masses and Polydispersity Obtained by Calibration with Pullulan Standards

integration limits	M_n/g mol^{-1}	M_w/g mol^{-1}	D
no tailing[a]	2.41×10^5	4.38×10^5	1.82
with tailing[a]	1.39×10^5	3.90×10^5	2.80

[a]See elution curves in the Supporting Information, Figure S4.

The molar mass and the dimensions of the zwitterionic polymer were investigated by static and dynamic light scattering at pH = 6.7. The respective Zimm plots are shown in Figure 1a−c for no added salt, 0.1 M $NaClO_4$, and 5 M $NaClO_4$ and are summarized in Table 2. The second virial coefficients are larger than 10^{-4} cm^3 mol g^{-2} which reveals even pure water to dissolve the polyzwitterion well as opposed to most polyzwitterions with betanoic side chains. The somewhat larger value of A_2 for salt-free solution may be caused by a few remaining net charges as discussed below.

The significantly smaller molar mass at no added salt, M_w = 5.6×10^5 g mol^{-1}, is most likely to be caused by some net charges and may be estimated by

$$1/P^{app} = 1/P_w + \alpha \quad (1)$$

with P^{app} the apparent degree of polymerization in saltfree solution, P_w the true weight-average degree of polymerization measured at high salt, and α the net degree of charge dissociation. From the data in Table 2 $\alpha \approx 0.01\%$. Thus, at pH = 6.7 the zwitterionic chain exhibits a tiny net charge in qualitative agreement with the determination of the isoelectric point (IEP) as discussed below.

The z-average diffusion coefficient determined by dynamic light scattering is obtained by extrapolation of the apparent diffusion coefficient D_{app} to zero scattering angle and zero concentration (see Supporting Information, Figure S3) according to[61]

$$D_{app} = D_z(1 + CR_g^2q^2)(1 + k_Dc) \quad (2)$$

with C a constant that depends on chain architecture and polydispersity.[61−63] k_D is related to the second virial coefficient A_2 and the concentration dependence of the friction coefficient k_f by[64]

$$k_D = 2A_2M_w - k_f - \nu \quad (3)$$

with ν the partial specific volume of the polymer. k_f was derived theoretically as[65−67]

$$k_f = k_{f0}N_AV_h/M_w \quad (4)$$

with N_A Avogadro's number, $V_h = (4\pi/3)R_h^3$ the hydrodynamic volume, and k_{f0} a parameter which depends on the interpenetration of two flexible chains and adopts values between 2.24 and 7.14, which are the limiting values for theta and good solvents, respectively.[66]

The values of k_{f0} determined for 0.1 and 5 M $NaClO_4$ solutions point toward a marginal solvent quality, whereas the somewhat higher value in water may be biased by subtle electrostatic effects. Because of considerable experimental uncertainty, the k_{f0} values should not be overinterpreted,

Table 2. Static and Dynamic Light Scattering Results for Poly(ε-*N*-methacryloyl-L-lysine) in Aqueous $NaClO_4$ Solution

solvent	R_g/nm	R_h/nm	ρ ratio	M_w/g mol^{-1}	A_2/mol cm^3 g^{-2}	k_d/cm^3 g^{-1}	k_{fo}	C
water	33	20	1.65	5.50×10^5	4.73×10^{-4}	291	6.23	1.29×10^{-1}
0.1 M	42	21.9	1.92	7.21×10^5	1.59×10^{-4}	83	3.96	1.39×10^{-1}
5 M	45	25.3	1.78	7.03×10^5	2.09×10^{-4}	148	2.5	1.51×10^{-1}

since they are derived from combination of four experimental quantities, all of which represent different averages given the broad molar mass distribution.

Both R_g and R_h expand a little bit upon addition of salt, but much less than typically observed for betanoic type of zwitterions. The ratio R_g/R_h scatters around 1.8 for all investigated salt concentrations, which may be interpreted in terms of polydisperse coils in good solvents in qualitative agreement with the large values of A_2 (see Table 2).

Because of the possibly significant influence of the excluded volume on the coil dimensions, the Kuhn statistical segment length l_k is difficult to determine. It may be estimated from R_g and R_h (the latter is known to depend less on excluded volume than R_g) according to the wormlike chain model.[68,69] Depending on salt concentration, the Kuhn statistical segment length is approximately l_k = 6−8 nm, which is quite large in view of the side chain length. However, CD spectroscopy did not reveal any secondary structure (results not shown) which could explain the pronounced chain stiffness. Most probably, repulsive dipolar and/or electrostatic interactions (see below for titration experiments) may be responsible for the stiffening.

In order to elucidate the effect of polyion charges on the zwitterion dimensions SLS and DLS measurements were performed at one small concentration in the range of 0.19 g/L > c > 0.13 g/L at different pH for 1.2 < pH < 12.2 at a constant $NaClO_4$ concentration of 0.1 M. The results are shown in Figure 2, where both R_g and R_h are seen to increase with addition of both HCl and NaOH but level off at pH < 3 and pH > 8. (For some representative static and dynamic light scattering data see Supporting Information, Figures S5−S9 and Table S1.)

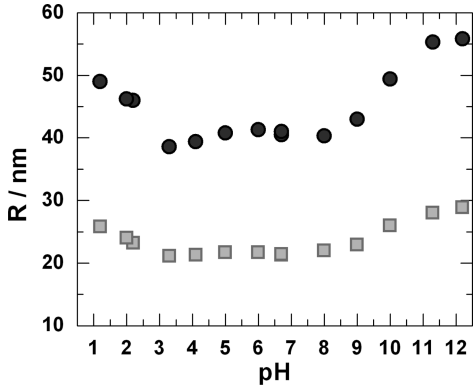

Figure 2. pH dependence of the radius of gyration R_g (circles) and the hydrodynamic radius R_h (squares) of poly(ε-*N*-methacryloyl-L-lysine) in 0.1 M $NaClO_4$.

Only at high (>9) and low (<3) pH, the dimensions increase moderately up to 40% as compared to those in the neutral solution. The effect is moderate because of two opposite trends: In order to induce a significant charge fraction on the zwitterion, a considerable amount of base and acid, respectively, has to be added which concomitantly increases the ionic strength, thus increasing the screening of electrostatic interactions. It was reported long ago[70] that the carboxy and amino groups in poly(ε-*N*-methacryloyl-L-lysine) exhibit only one pK_a value around 6.5, which does not depend on the amount of added salt. This is in contrast to the behavior of polycarboxybetaines which show two pK_a values which drift further apart upon addition of salt.[71] It is concluded that the close spatial neighborhood of carboxy and amino groups drastically influences the acid−base equilibrium due to strong electrostatic interactions. Such behavior was theoretically predicted long ago by pioneering works of Rice and Harris[72] and Katchalsky.[73]

Potential Application in Biological Systems. Zwitterionic polymers are known for their biocompatibility,[47,74] antifouling properties,[56,57] and low protein adsorption from human blood.[48,53−55] Therefore, poly(ε-*N*-methacryloyl-L-lysine) might be a suitable candidate as a "nano carrier" for biomedical applications like gene transfection or targeted delivery of drugs. The requirements for such a nano carrier are low toxicity and no severe interactions with biological media like blood serum, which in the worst case could lead to the formation of large aggregates. In addition, sufficient functional groups should be available for either complex formation with DNA or for conjugation of drugs, antibodies, etc.

1. Toxicity. In order to determine the toxicity of the polyzwitterion, two cell lines were utilized: bone marrow-derived dendritic cells (BM-DC) and human embryonic kidney 293 cells (HEK293). The results are summarized in Table 3 and show no significant toxicity at concentrations as high as 1 mg/mL. Since "in vitro" and "in vivo" experiments are conducted at much lower concentrations, we did not investigate even higher concentrations.

2. Aggregate Formation in Human Serum. Recently, our group developed a new method based on dynamic light scattering to determine the aggregation of nanoparticles in human blood serum.[75] Briefly, the correlation function of nanoparticles or polymers in serum solution, $g_1(t)_{mix}$, should be well fitted the appropriately weighted sum of the known

Table 3. Viability (v) of Bone Marrow-Derived Dendritic Cells (BM-DC) and Human Embryonic Kidney 293 Cells (HEK293) in the Presence of Polymer Solutions with Different Concentrations

	c(polymer)/μg μL^{-1}				
	0.01	0.05	0.1	0.5	1
v(BM-DC)/%	78.2 ± 3.8	74.9 ± 4.1	80 ± 5.3	78.2 ± 9	78.3 ± 3.6
v(HEK293)/%	97.9 ± 3.1	86.3 ± 6.2	72.6 ± 5.2	77.8 ± 4.4	74.7 ± 3.7

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correlation functions measured from the polymer in isotonic solution, $g_1(t)_p$, and of undiluted serum, $g_1(t)_s$

$$g_1(t)_{mix} = a_p g_1(t)_p + a_s g_1(t)_s \quad (5)$$

with the amplitudes a_p and a_s the only fit parameters. Such a fit is shown in Figure 3 for poly(ε-*N*-methacryloyl-L-lysine) in undiluted serum. The lack of significant systematic residues demonstrates aggregate formation of poly(ε-*N*-methacryloyl-L-lysine) with the complex protein mixture in serum to be negligible.

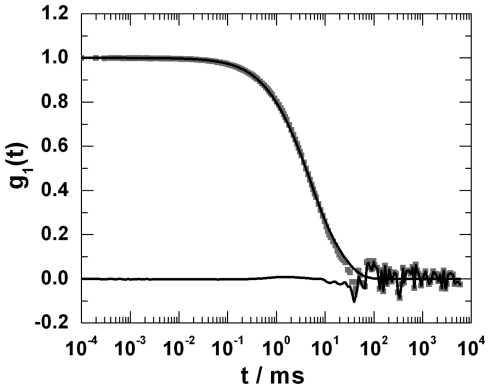


Figure 3. Correlation function of poly(ε-*N*-methacryloyl-L-lysine) in serum (squares) and force fit (line) according to eq 5. Scattering angle 30°.

3. Conjugation with Alexa Fluor 488 Dye. In another preliminary experiment the dye Alexa Fluor 488 was conjugated to the zwitterion according to Scheme 2. Capillary electrophoresis (see Supporting Information Figure S10) showed that excess dye was removed quantitatively by dialysis using Amicon Ultra centrifugal filter devices (3 kDa). FCS confirmed this result and additionally revealed that about 10 dye molecules are chemically bound to one zwitterion chain as estimated by comparing the fluorescent brightness of individual Alexa 488 molecules and labeled polymer chains (Figure 4).

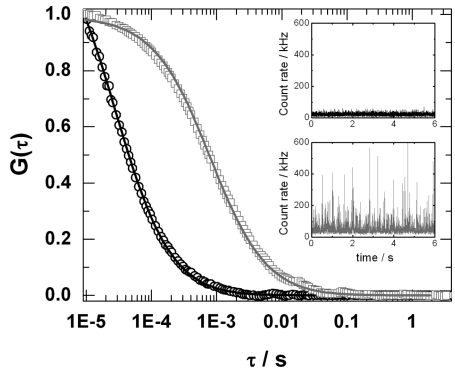
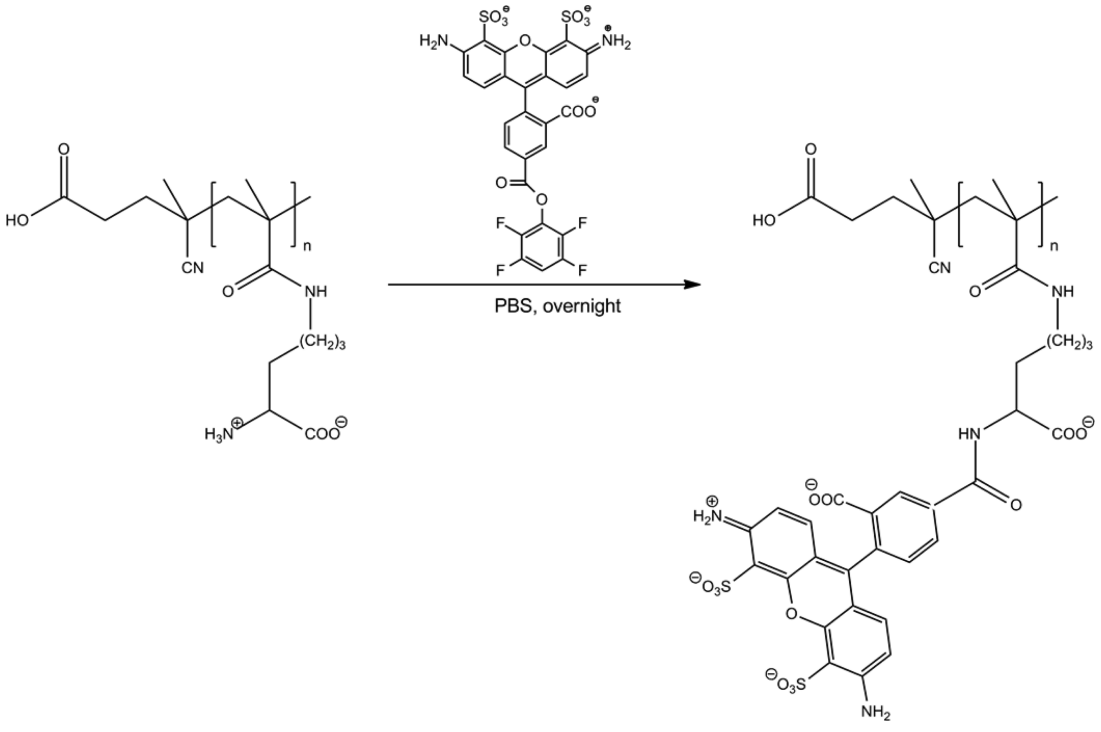


Figure 4. Normalized FCS autocorrelation functions and the corresponding fits (lines) according to eq S1 for Alexa Fluor488 (black circles) and dye-labeled poly(ε-*N*-methacryloyl-L-lysine) (gray squares). The upper and lower insets show the respective fluorescence intensity traces measured in solutions with similar molar concentrations.

Isoelectric focusing by a pH gradient in solution and in a gel consistently yielded a very low IEP = 3.6 ± 0.3 (see Supporting Information, Figures S11, S12 and Table S2) which is much lower than reported for the IEP of the unmodified zwitterion.[70] Therefore, we examined the IEP for unmodified polyzwitterion by zeta-potential measurements. The results are shown in Figure 5 and qualitatively confirm the literature value of IEP = 6.4. Zeta-potential measurements of the dye-labeled polyzwitterions yielded qualitatively different results as compared to the unlabeled samples but did not allow for an unambiguous

Scheme 2. Labeling of Poly(ε-*N*-methacryloyl-L-lysine) with Alex Fluor 488



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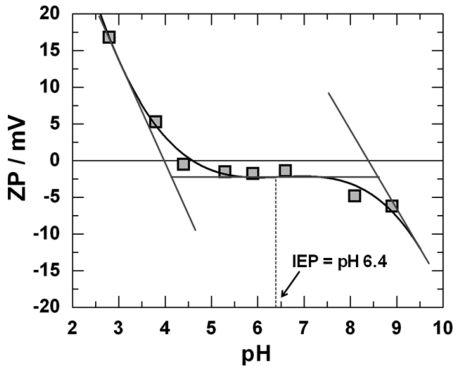

Figure 5. Determination of the isoelectric point of unlabeled poly(*ε*-*N*-methacryloyl-L-lysine) by zeta-potential measurements. The small negative offset (−2 mV) at the IEP is most likely due to experimental uncertainty of the instrument.

determination of the IEP (see Supporting Information, Figure S13).

Apparently, the IEP of the dye-labeled polymer is dominated by the IEP of the bound dye, which has been observed for other proteins as well.[76−78]

At neutral pH the dye-labeled zwitterion should have a significant negative charge, which should inhibit cell uptake. Preliminary FACS measurements indeed reveal an extremely small uptake of 1.8% by bone marrow dendritic cells (see Figure 6). Such a small unspecific uptake is mandatory for future experiments aiming at antibody mediated uptake, i.e., cell targeting.

4. DNA Complexation. Eventually, the complex formation of poly(*ε*-*N*-methacryloyl-L-lysine) with DNA was investigated. Based on the reported IEP, the zwitterion should not exhibit a cationic, but rather a small anionic, charge at neutral pH. Accordingly, a test experiment revealed no complex formation with DNA. Since polymers with carboxylate groups in the side chain like poly(acrylic acid) are known to form strong complexes with divalent metal ions like Ca^{2+} or Zn^{2+}, a net cationic charge ought to be created if the carboxylate groups of the zwitterion would bind divalent metal ions.[79,80] As opposed to poly(acrylic acid), the binding of the zwitterionic polymer with Ca^{2+} or Zn^{2+} does not lead to aggregation/phase separation at neutral pH in the presence of 0.15 M NaCl for Ca^{2+}/COO^{-} < 115 or Zn^{2+}/COO^{-} < 50 (see Supporting Information Figure S14). Higher ratios were not investigated. Next, the complex formation of the polyzwitterion with DNA in the presence of Ca^{2+} and Zn^{2+} was investigated. Whereas some DNA complex formation could be detected in the presence of very high Ca^{2+} concentration, the zwitterion did form complexes with DNA in the presence of Zn^{2+} if Zn^{2+}/COO^{-} > 10. Examples are shown in Figure 7 where the

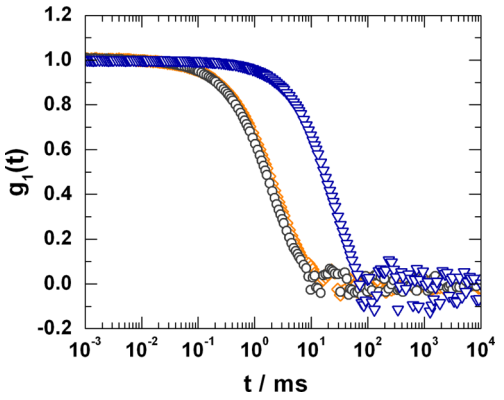

Figure 7. Correlation functions of poly(*ε*-*N*-methacryloyl-L-lysine) in aqueous 0.15 M NaCl solution (orange diamonds), polymer + Zn^{2+} (Zn^{2+}/zwitterionic repeat unit = 16) (gray circles), polymer + Zn^{2+} + DNA (PO_4^{-}/zwitterionic repeat unit =0.31) (blue triangles). Scattering angle 30°.

correlation functions clearly revealed complex formation if the Zn^{2+}/PO_4^{-} ratio is high enough. This ratio seems to decrease with increasing Zn^{2+}/COO^{-} ratio as expected (results not shown). Evaluation of the hydrodynamic radii yields a small shrinkage of the polyzwitterion from R_h = 23 nm to R_h = 20 nm upon addition of Zn^{2+}. Subsequent addition of DNA resulted in large complexes of R_h = 220 nm. It should be noted that neither Ca^{2+} nor Zn^{2+} yields intermolecular aggregates with DNA for Ca^{2+}/PO_4^{-} < 157 and Zn^{2+}/PO_4^{-} < 84; larger ratios were not measured yet. A detailed investigation of DNA complex formation of the polyzwitterion in the presence of divalent metal ions is currently performed, and the suitability of such complexes for gene transfection is elucidated. The results will be subject of a future communication.

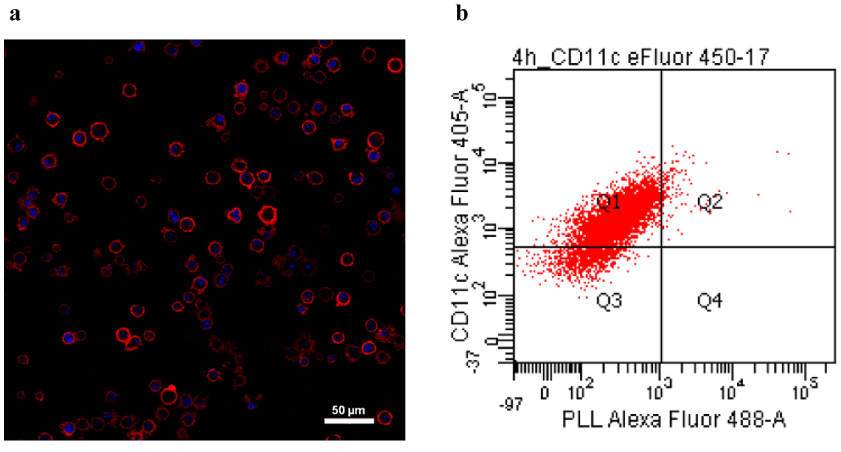

Figure 6. Cell uptake of Alexa Fluor 488-labeled poly(*ε*-*N*-methacryloyl-L-lysine) by bone marrow dendritic cells determined by (a) CLSM images and (b) FACS measurements. Cells in 1xPBS, nonfixated, incubation time 4 h, concentration 1×10^{12} particles per 5×10^{5} cells. Blue: Hoechst 33342 labeling of nucleus; red: cell mask; orange: labeling of cell membrane; green: poly(*ε*-*N*-methacryloyl-L-lysine)-AF488.

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CONCLUSION

Despite their favorable biocompatibility, the application of polyzwitterions, particularly of polybetaines, in drug delivery is problematic due to the poor solubility in water and in isotonic aqueous solution. The present work elucidates in some detail the solution properties of a long known hydrophilic polyzwitterion consisting of ε-*N*-methacryloyl-L-lysine repeat units.

This polyzwitterion was demonstrated to fulfill four major requirements for potential "in vivo" applications: little cytotoxicity, no aggregate formation in human serum, multifunctional conjugation sites, and no unspecific uptake in bone marrow dendritic cells. Particularly the latter two properties make them ideal candidates for antibody conjugation leading to antibody-mediated specific cell targeting.

The complexation behavior toward divalent metal ions such as Ca^{2+} or Zn^{2+} is strikingly different from that of poly(methacrylic acid) in that metal ion-mediated intermolecular aggregation/phase separation is effectively suppressed. Still, the complexation with the carboxylate groups of the polyzwitterion with Zn^{2+} ions appears to be strong enough in order to induce a cationic net charge which is sufficiently strong to promote complexation with plasmid−DNA. Future experiments will show whether the stability of the complexes upon dilution of the Zn^{2+} ions is favorable for appropriate DNA release for "in vivo" and "in vitro" transfection. The potential advantage of such a system could be that the residual components after DNA release; i.e., Zn^{2+} ions and polyzwitterions are most likely nontoxic.

ASSOCIATED CONTENT

Supporting Information

NMR spectra, HPLC and GPC elution curves, q^2 dependence of reduced scattering intensities and diffusion coefficients, correlation functions, and IEP determination. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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