Novel Amphoteric Cystine-Based Poly(amidoamine)s Responsive to Redox Stimuli

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ABSTRACT: Novel poly(amidoamine)s (PAAs) containing disulfide linkages regularly arranged along the polymer chain, namely BP−CY and BAC−CY, were synthesized by stepwise polyaddition of L-cystine to 1,4-bis(acryloyl)-piperazine (BP) and 2,2-bis(acrylamido)acetic acid (BAC), respectively. Even if L-cystine contains four acid hydrogens, no evidence of cross-linking was found. All products were characterized by ^{1}H and ^{13}C NMR spectroscopy, and their average molecular weight determined by size exclusion chromatography. The polymerization rates were investigated by means of ^{1}H NMR spectroscopy. In both cases, the experimental data were consistent with pseudo-second-order kinetics. The calculated kinetic constants were $k_{c,BP} = 8.10 \times 10^{-3}$ min^{-1} L mol^{-1} and $k_{c,BAC} = 1.41 \times 10^{-3}$ min^{-1} L mol^{-1} for the polyaddition of L-cystine to BP and BAC, respectively. A potentiometric study was carried out of BP−CY and BAC−CY speciation as a function of pH, and the electrochemical activity of their disulfide bonds as a function of pH was investigated by cyclic voltammetry on hanging drop mercury electrode, revealing many analogies with parent L-cystine. BP−CY and BAC−CY degraded in aqueous systems at a rate similar to that usually reported for PAAs. In the presence of reducing agents, however, they degraded completely in a few minutes. Preliminary biocompatibility in vitro tests showed that both BP−CY and BAC−CY are devoid of appreciable toxicity.

Introduction

Considerable interest is being focused on soluble synthetic polymers stable in the bloodstream, but degradable after internalization in cells. Typical examples are biocompatibile polymers bearing disulfide linkages in the main chain, which can be regarded as stimuli-responsive release systems where the release of a drug payload can be triggered by reductive cleavage of the disulfide linkages with consequent polymer chain degradation. These systems can be specifically designed for intracellular delivery thanks to the stronger reducing environment within cells than in extra-cellular fluids. In oral administration, disulfide linkages permit delivering the active substance to the colon, where reduction is caused by change in bacterial flora, while they are not affected by the pH change in the digestive tract.[1,2] Different authors have proposed PEG conjugates with bioactive molecules, as for instance peptides or enzymes,[3,4] drugs,[5] or liposomes,[6,7] in which the PEG−substrate linkage was a disulfide group, which proved stable at neutral pH, but unstable in physiologically relevant reducing environments.

Poly(amidoamine)s (PAAs) are a family of synthetic polymers characterized by the presence of *tert*-amine and amide groups regularly arranged along the backbone. The first studies on PAAs were published around 1970.[8] Subsequently, their synthesis and properties have been reviewed in several instances.[9,11,12] PAAs are normally degradable in aqueous environments[13,14] and most of them are only moderately toxic, in spite of their polycationic nature.[9−16] Amphoteric PAAs, carrying carboxyl groups as side substituents, are even less toxic and may be approximately as biocompatible as dextran. The same PAAs when injected in animals are endowed with "stealth" properties, and passively concentrate in solid tumors by the EPR (enhanced permeation and retention) effect.[17] Both amphoteric and non-amphoteric PAAs have shown potential as endosomolytic polymers for the delivery of genes and toxins.[17,18]

PAAs are obtained by Michael-type polyaddition of primary monoamines or bis(*sec*-amines) to bis(acrylamide)s. The reaction is remarkably specific and leads to a large variety of polymer structures. For instance, in a previous paper we have reported on the preparation and properties of novel poly(amidoamine)s (PAAs) bearing disulfide linkages along the polymer chain by reaction of 2-methylpiperazine with *N*,*N*′-bis(acryloylcystamine) and with *N*,*N*′-bis(acryloylcystine).[19]

As a rule, hindered amines polymerize slowly with bis(acrylamide)s to high molecular weight polymers.[9,10] Primary aminoacids carrying no substituents in the α position, such as glycine, β-alanine and the like, polymerize with bis(acrylamide)s as any other primary monoamine. By contrast, natural α-aminoacids other than glycine do not yield polymers under the usual preparation conditions for PAAs.[8,9] This anomalous behavior can be explained by a combination of two factors. First, for all

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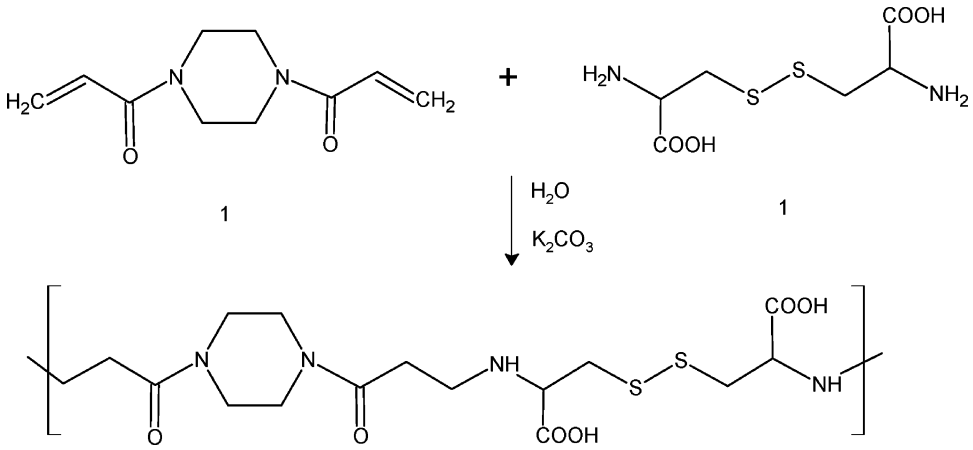
Scheme 1. Synthesis of BAC−CY from L-Cystine and BAC



primary amines the rate of the second addition step to acrylamides is much lower than that of the first step. This is due to increased steric hindrance and decreased nucleophilicity of the addition product compared to the parent amine, as a consequence of the introduction of a substituent carrying an electron-withdrawing group in the β-position. For instance, the reaction constant of the second addition step of the amine groups of poly-L-lysine to *N*,*N*-dimethylacrylamide was lower by more than 1 order of magnitude than that of the first one.[20] Second, for α-aminoacids the reactivity of the amine groups is additionally biased by the presence on the α carbon of the bulky carboxylate group. These considerations lead us to envisage that any compound carrying two α-substituted aminoacid moieties would behave as a difunctional monomer in PAA synthesis. In particular, using L-cystine as monomer could provide a straightforward route to disulfide-containing PAAs.

On the basis of this premise, here we present two novel linear amphoteric PAAs carrying disulfide linkages in the main chain, obtained by the polyaddition of L-cystine to 1,4-bis(acryloyl)-piperazine and 2,2-bis(acrylamido)acetic acid.

Experimental Part

Materials. L-Cystine (≥99.0%), potassium carbonate (99%), and 1,4-dithio-D,L-threitol (99%) were purchased from Fluka and used without further purification. D_2O (99.9%), stabilized over silver coil were purchased from Aldrich. 2,2-Bis(acrylamido)acetic acid (BAC)[21] and bis(acryloyl)piperazine (BP)[22] were synthesized as previously described. The volumetric HCl aqueous solutions were purchased from Rieden de Haen.

Cell line 3T3/BALB-c Clone A31 mouse embryo fibroblasts (CCL163) was obtained from the American Type Culture Collection (ATCC) and propagated as indicated by the supplier.

Dulbecco's Modified Eagles Medium (DMEM), 0.01 M pH 7.4 phosphate buffer saline without Ca^{2+} and Mg^{2+} (PBS), fetal bovine serum (FBS), trypsine/EDTA, glutamine, and antibiotics (penicillin/streptomycin) were purchased from GIBCO Brl. Cell proliferation reagent WST-1 was purchased from Roche Diagnostic. Tissue culture grade disposable plastics were obtained from Corning Costar.

Instruments and Methods. The ^1H and ^{13}C spectra were acquired on a Brüker Avance 500 spectrometer, operating at 500.133 MHz (^1H) and at 125.00 MHz (^{13}C).

Size exclusion chromatography (SEC) traces were obtained making use of TSK-gel G4000 PW and TSK-gel G3000 PW columns produced by TosoHaas. The two columns were connected in series and the mobile phase was Tris buffer pH 8,10; flow rate 1 mL/min (Waters model HPLC pump 515); the UV detector was a Waters model 486, operating at 230 nm; the refractive detector was a Waters model 2410. The samples were prepared in Tris buffer with a 1% concentration in polymer. Molecular weight determinations were based on a calibration curve obtained with pullulan standards.

For routine culturing and evaluation of morphology, cells were analyzed under an inverted microscope Nikon Eclipse TE2000-U.

Polymerization of L-Cystine with BP (BP−CY). In a round bottomed flask, equipped with a magnetic stirrer and nitrogen inlet, L-cystine (1.00 g, 4.12 mmol) was dissolved under inert atmosphere in bidistilled water (1.4 mL), together with potassium carbonate (1.115 g, 8.24 mmol). BP (0.818 g, 4.12 mmol) was added, and the reactive solution was left under stirring for 30 min. Then the nitrogen inlet was closed and the reaction vessel placed in a thermostatic bath at 25 °C, and protected from direct light. After 48 h the viscous solution was diluted with a 1 M HCl aqueous solution down to pH 3.0 and then precipitated with 3:1 v/v acetone. The precipitate was extracted by a further two volumes of acetone and eventually dried in vacuo. Yield: 1.98 g (73.8%, calculated after the composition of the product by elemental analysis). $\bar{M}_n$ = 5000; $\bar{M}_w$ = 13 500.

Elemental Analyses. Anal. Calcd for ($C_{14}H_{22}N_4O_6S_2 \cdot 2H_2O$, 0.2HCl): C, 40.17; H, 6.32; N, 11.71; S, 13.40; Cl, 1.60. Found: C, 39.73; H, 5.90; N, 11.57; S, 13.76; Cl, 1.63.

Solubility. The polymer is soluble in aqueous media at all pH values and DMSO but insoluble in most organic solvents.

Polymerization of L-Cystine with BAC (BAC−CY). The same procedure described for BP−CY was followed, but substituting BAC (1.02 g, 4.12 mmol) for BP and using a 1.5-fold amount of potassium carbonate. The final product was recovered by acidifying down to pH 3.0. The precipitate was extracted by further two volumes of acetone and dried to constant weight in vacuo. Yield: 1.576 g (74.7%, calculated after the composition of the product by elemental analysis). $\bar{M}_n$ = 11 900; $\bar{M}_w$ = 51 800.

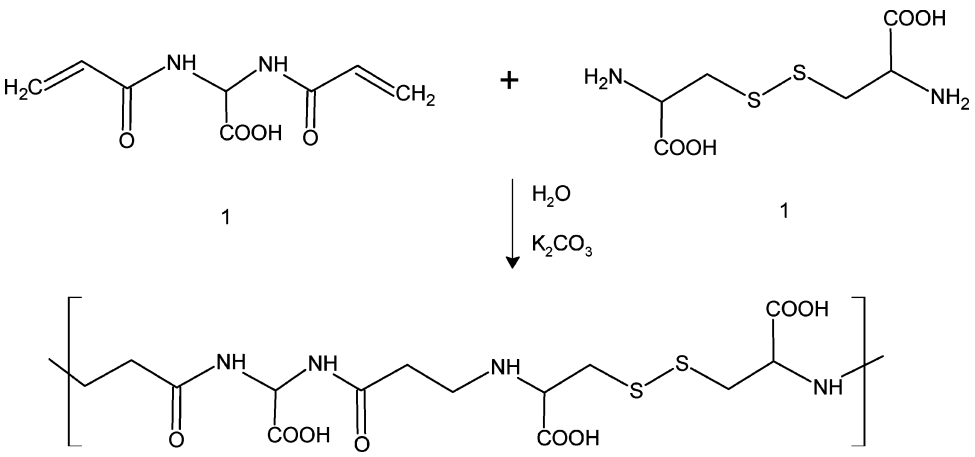
Elemental Analyses. Anal. Calcd for ($C_{12}H_{18}N_4O_8S_2$ $2H_2O$ HCl): C, 30.19; H, 4.89; N, 10.06; S, 11.51; Cl, 6.90. Found: C, 33.59; H, 5.35; N, 9.36; S, 10.82; Cl, 7.60.

Solubility. The polymer is insoluble in aqueous media in the pH interval 1.0−4.0, whereas it is soluble at all other pH values. It is insoluble in organic solvents.

NMR Measurements. All spectra were performed with a Brüker Avance 500 spectrometer at room temperature, in H_2O as solvent, in the presence of a sealed capillary containing D_2O for looking and tetramethylsilane (TMS) as internal reference. $^{13}C\{^1H\}$ spectra were obtained using Waltz decoupling and were exponentially multiplied to give 0.8 Hz line broadening before Fourier transformation. The JMod spectra were recorded using standard Brüker software sequences.

2D Spectra. All two-dimensional experiments were acquired with a Brüker inverse 5 mm z-gradient probe. The 90° pulse widths were 6.6 and 12.83 μs for ^1H and ^{13}C, respectively. The gradient was shaped by a waveform generator and amplified by a Brüker B-AFPA-10 amplifier. A sinusoidal gradient of 1 ms length and a recovery time of 0.1 ms was used. The 2D COSY spectra were

Scheme 2. Synthesis of BP−CY from L-Cystine and BP



recorded with a 1024 × 1024 data matrix and 512 increments of 1 scan each, in magnitude mode, with a relaxation delay of 6.0 s and using a 1:1 gradient combination. The HMQC and HMBC spectra were recorded using standard Brüker software sequences inv4gs and inv4gslplrnd, respectively. The following acquisition parameters were applied in both experiments: spectral widths in f_1(^{13}C) and f_2(^{1}H) dimensions 20 000 and 4000 Hz, respectively, a 1024 × 1024 data matrix, 256 time increments of 520 scans each and a 5:3:4 gradient combination.[23,24]

Kinetic Study of the Polymerization Reaction of L-Cystine with BP. L-Cystine (23.8 mg, 0.10 mmol), K_2CO_3 (29.3 mg, 0.21 mmol) and BP (20.2 mg, 0.107 mmol), were dissolved in D_2O (0.75 mL) under nitrogen atmosphere and *tert*-butanol (10 μL) was added as internal standard. This reactive mixture was rapidly transferred into an NMR tube and the tube sealed. The experiment was carried out at 25 °C. The progress of the polymerization reaction was followed by monitoring the decrease of the peak integral of the double bond hydrogens (5.5 and 6.8 ppm) of bis(acrylamide) with respect to that of *tert*-butanol CH_3 groups (1.15 ppm).

The kinetics of the polymerization reaction of L-cystine with BAC was studied as described for BP. The amounts of reagents used were as follows: L-cystine (24.3 mg, 0.10 mmol), K_2CO_3 (41.85 mg, 0.30 mmol), BAC (20.69 mg, 0.10 mmol), D_2O (0.75 mL) and *tert*-butanol (10 μL).

Both kinetic experiments were repeated at higher concentrations (0.8 M in both monomers), by following the same procedure.

Potentiometric Determination of the Polymer Acid Dissociation Constants. BP−CY and BAC−CY samples (about 0.0025 M in highly deionized water [Millipore Milli-Q system], with the addition of 0.1 M NaCl (Fluka Biochemika Microselect) as an ionic strength stabilizer), deaerated by continuous ultrapure N_2 bubbling, and thermostated at 25 °C, have been pH-metrically titrated forward and backward with 0.1 M NaOH and HCl Merck standard solutions. The operating cell (including a 211/S6G/12 AMEL glass electrode and an AMEL 3842 saturated calomel electrode with a double bridge filled with 0.1 M KNO_3, connected to an AMEL 631 differential electrometer) was calibrated over a wide pH range using a multiple pH standard buffer set, including 0.05 *m* potassium tetraoxalate [pH 1.68]; three Certipur Merck buffers at pH 4.0, 7.0 and 9.0; and 0.025 *m* $NaHCO_3$ + 0.025 *m* Na_2CO_3 [pH 10.01]. Moreover, the calibration was checked and refined by closely fitting theoretical titration curves, calculated by the nonapproximated De Levie treatment of multiple acid/base equilibria,[25] to experimental forward- and back-titration curves of 0.175 *m* H_3PO_4 (Fluka Biochemika), having three dissociation constants very similar to the target polymer ones. The same modelization was afterward applied to the simulation of the experimental BP−CY and BAC−CY titration curves, leading to optimization of the sought pK_a values. The flat portion of the titration curves corresponding to the buffer region was also submitted to the approximated treatment by Katchalsky and Spitnik,[26] in order to evaluate the β parameter accounting for possible nonequivalence of identical functional groups located on adjacent monomeric units.

Hydrolytic Degradation Studies. The hydrolytic degradations of BP−CY and BAC−CY were followed viscometrically in a 0.1 M $NaHCO_3$ buffer solution at pH 8.3, and in a 0.1 M HCl aqueous solution at pH 1−4. The polymer sample (0.100 g) was dissolved in 25 mL aqueous solution pre-thermostated at 37 °C in an external bath. The solution obtained was then rapidly placed in a Ubbelhode viscometer maintained at 37 °C and the decrease of the reduced viscosity monitored with time.

Reductive Degradation Studies. The reductive degradation was followed viscometrically through the same procedure described for the hydrolytic degradation. However, after a first time flow measurement on polymer solutions with the same concentration used in the hydrolytic degradation, 1,4-dithio-D,L-threitol was added in a 1.1/1 mol/mol ratio with respect to the disulfide functions in the polymer sample.

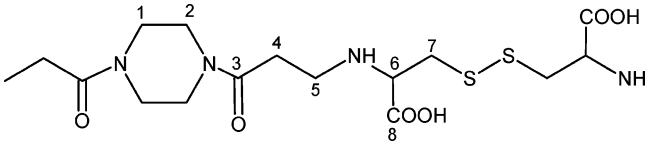
Voltammetric Studies. The voltammetric investigations were carried out in a cell thermostated at 25 °C, on solutions deareated by chromatography-grade nitrogen bubbling, made up in ultrapure water, with 0.1 M tris(hydroxymethyl)aminomethane (TRIS, NIST Standard Reference Material 922) as a pH buffer and the supporting electrolyte. The substrate concentration was 0.0005 M both in the case of cystine and of the polymers' disulfide units. The operating pH was modulated by small additions of HCl (37% Carlo Erba ISO for analysis). The CV characteristics were obtained using an Autolab PGSTAT 12 potentiostat/galvanostat (EcoChemie, The Netherlands) run by a PC with GPES software, at scan rates ranging 0.02−5 V s^{-1} and at different pH, using a hanging drop mercury electrode (HDME WK 2, Institute of Physical Chemistry, Polish Academy of Science) as the working electrode, a platinum counter electrode, and an aqueous saturated calomel electrode (SCE) as the operating reference electrode.

Cell Line. Cytoxocity evaluations of the investigated materials were carried out using the 3T3/BALB-c Clone A31 cell line. Cells were grown in DMEM containing 10% FBS, 4 mM of glutamine, and 100 U/mL:100 μg/mL penicillin:streptomycin (complete DMEM).

Subculturing. A 25 mL flask containing exponentially growing 3T3 cells was observed under and inverted microscope for cell confluence. The complete DMEM media was then removed, and cells were rinsed for a few min with PBS. The buffer solution was removed, and cells were incubated with 0.5 mL of trypsin/EDTA solution at 37 °C in 5% CO_2 incubator for 5 min or until the monolayer started to detach from the flask. Cells were suspended in an appropriate volume of DMEM and plated at a split ratio of 1:6 or 1:10 in a 75 mL flask.

Determination of IC_{50} of Polymeric Materials. The IC_{50} (50% inhibitory concentration, that is the material concentration at which 50% of cell death in respect to the control is observed) of the investigated polymers was evaluated by exposing cells for 24 h to DMEM containing different polymer concentrations. At the end

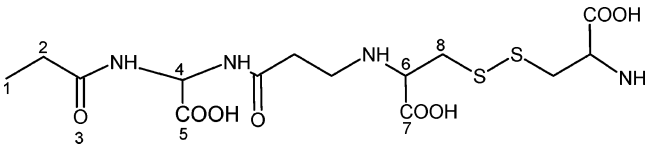
Table 1. Relevant ^{1}H and ^{13}C Chemical Shifts (δ) of BP−CY



	1−2	3	4	5	6	7	8
^{1}H	3.5−3.6		2.92	3.36	4.02	3.23; 3.40[a]	
^{13}C	41.4−44.65 41.2−44.5	170.7	28.4	43.3	60.2	36.8	171.1

[a] AB system.

Table 2. Relevant ^{1}H and ^{13}C Chemical Shifts (δ) of BAC−CY



	1	2	3	4	5	6	7	8
^{1}H	3.27	2.69		5.71		4.33		3.27
^{13}C	42.6	30.4	171.8	56.2	170.4	58.5	169.3	35.2

of the exposure time, cells were incubated with WST-1 cell proliferation reagent for the quantitative evaluation of cell proliferation.

Cell Proliferation Assay. Quantitative proliferation was assayed by using the cell proliferation reagent WST-1 and by following the protocol indicated by the manufacturer. Briefly, cells were allowed to proliferate in DMEM containing different concentration of polymeric materials and then incubated or 4 h with an appropriate volute of WST-1 tetrazolium salts. Formazan production was detected at 450 nm, with 620 nm as reference wavelength using and ELISA microplate reader (Biorad)

Results and Discussion

Synthetic Considerations. This paper reports on two novel amphoteric PAAs obtained by polyaddition of L-cystine to BP (Scheme 1) and BAC (Scheme 2).

Both BP−CY and BAC−CY preparations are exceptional in the PAA domain, in that they involve primary diamines and yet give linear polymers. As pointed out in Introduction, natural α-aminoacids other than glycine do not give polymers with bis(acrylamide)s under the conditions usually employed for PAAs, owing to the low reactivity of the product of a first addition step toward a second addition. This gave us the rationale for employing L-cystine in the same way as a bis(*sec*-amine) in PAA synthesis.

The polymerization reaction leading to PAAs is generally carried out in solvents carrying acid hydrogens, such as water or alcohols, with no added catalysts. In the present investigation, both cystine-containing PAAs were obtained in aqueous solution at a 1.5 M concentration in both monomers.

Using carboxylated bis(acrylamide)s and aminoacids as monomers, enough base must be added to the monomer mixture to ensure that the amine groups involved in the polyaddition reaction are not protonated. Very strong bases such as lithium or sodium hydroxides posed in this work a practical problem of dosage because in both polymerizations even the slightest excess was apt to trigger degradation of the disulfide bond. Therefore, we preferred to add 1 mol of K_2CO_3 per mole of carboxyl group, considering that the resultant systems were self-buffering. By this procedure, the polymerization proceeded as usual for PAAs. The products were isolated in acidic form, which is considered to be more stable, and were stored under dry atmosphere.

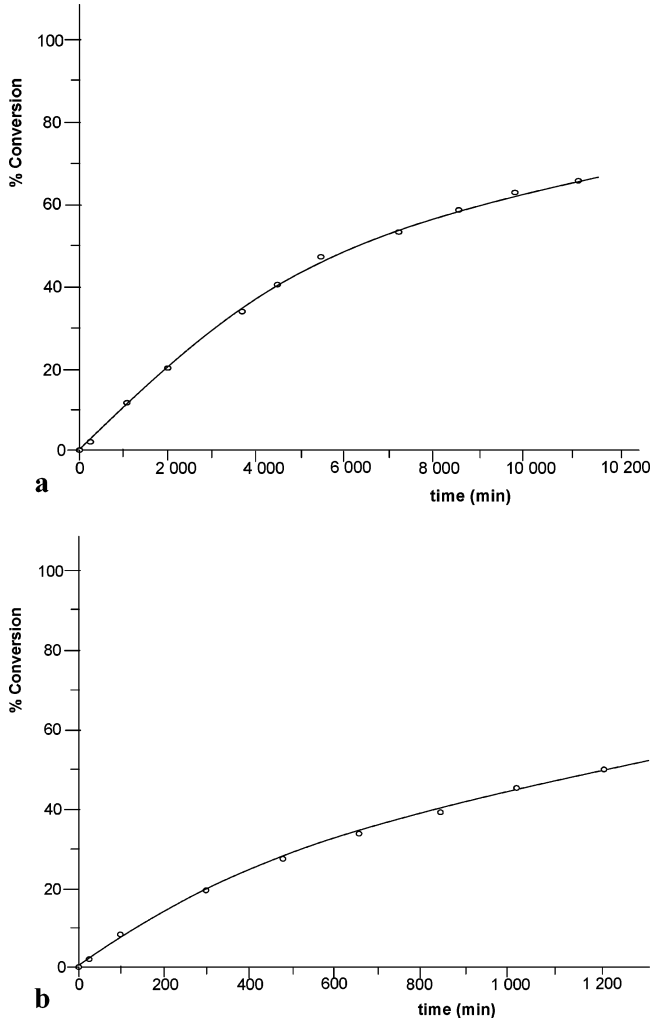


Figure 1. Kinetics of the polymerization reaction of L-cystine with activated bis(acrylamide)s in D_2O, monomers' concentration = 0.1 M, and at 25 °C. Panel a: reaction of L-cystine and BAC. Panel b: reaction of L-cystine and BP. Solid lines: calculated curve. Circles: experimental data.

NMR Characterization of BP−CY and BAC−C. As previously reported,[27] the characterization procedure involved a concerted use of 1D and 2D NMR spectroscopy. In particular, together with the ^{1}H, ^{13}C, and Jmod 1D NMR spectra, several 2D gradient-enhanced experiments such as 2D COSY, ^{1}H/^{13}C 2D HMQC (heteronuclear multiple-quantum coherence) and ^{1}H/^{13}C 2D HMBC (heteronuclear multiple-bond correlation) were performed.[23,24]

The data relative to BP−CY and BAC−CY will be discussed separately. A feature common to both polymers was the absence of residual terminal double bonds.

BP−CY. The ^{1}H and ^{13}C spectra in water of BP−CY as obtained (see the Experimental Part) revealed at once the presence in the repeating units of three components, giving three different very close sets of resonances **(a**, **b**, and **c)** in 80:13:7 ratio. The ^{1}H and ^{13}C complete assignments are reported, for the major component **a**, in Table 1.

The ^{13}C Jmod spectrum revealed that the methynic carbon atom (C-6) is present in **a**, **b**, and **c**, at 60.2, 53.1, and 58.2 ppm, respectively. Indeed, from the ^{1}H/^{13}C HMQC map we found for each component the corresponding correlated H-6 protons at 4.02, 4.1, and 3.98 ppm, respectively, and from the

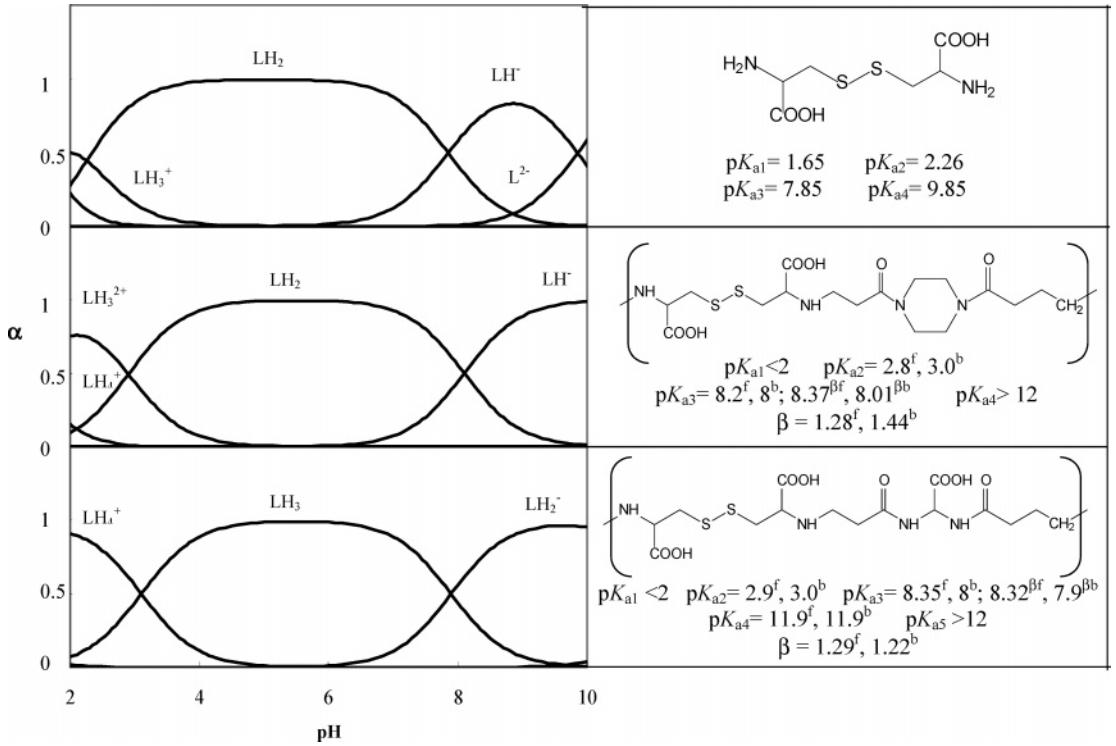


Figure 2. pK_as and speciation diagrams obtained for BP−CY and BAC−CY, plus parent L-cystine, from the De Levie treatment of their forward (f) and backward (b) pH-metric titrations, or the classical approximate approach hinging on the β parameter (β).

^{1}H−^{1}H COSY the coupling of each H-6 with the AB system of its own H-7 hydrogens. With the support of the ^{1}H/^{13}C 2D HMBC experiments it was possible to assign the H-4 and H-5 protons, at 2.92 and 3.36 ppm, respectively, the same for **a**, **b**, and **c**, by the long-range coupling constant between the carbonyl C-3 and H-4. The attributions of H-1, C-1 and H-2, C-2, showing the same resonances in **a**, **b**, and **c**, were then obvious.

A drop of dilute HCl was then added to the polymer's solution. After that the ^{1}H spectrum revealed that compound **c** was no longer present, while the **a/b** ratio was 88:12. We could reasonably deduce that compound **c** corresponded to incompletely protonated units that turned to **a** on further acidification. Comparing the ^{13}C assignments for components **a** and **b**, we noticed that exclusively the C-6 and C-7 carbons showed a noticeable chemical shift variation. In the ^{13}C spectra, for instance, passing from **a** to **b** their chemical shifts changed from 60.2 and 36.8 ppm to 53.1 and 37.8 ppm, respectively. This observation allowed us to hypotesize the presence in **b** of SH terminal functions, resultant from disulfide bond cleavage.

BAC−CY. At strongly acidic pH, two components were present also in the case of BAC−CY in approximately 92/8 ratio. As regards the major component, by the above-described procedure we could recognize the expected resonances, and confirm the 1:1 ratio between BAC and cystine moieties. The assignments were performed using the correlations found in the ^{1}H−^{1}H COSY, ^{1}H/^{13}C HMQC and HMBC experiments, as in the case of BP−CY (see Table 2).

In BAC−CY, similarly to BP−CY, passing from the major to the minor component the chemical shifts of C-6 and C-8 carbons changed from 58.5 and 35.2 ppm to 51.48 and 36.1 ppm, respectively. We think that also in this case the minor derivative carries an SH terminal function.

Polymerization Kinetics. The polyaddition kinetics of L-cystine with BP and BAC were investigated by ^{1}H NMR spectroscopy in D_2O solutions, following the decrease of the bis(acrylamide) double bond resonances, as described in the Experimental Part. The reactions were run directly in the NMR tube at two different concentrations, 0.1 and 0.8 M. These values were lower than in standard PAA preparations so as to ensure lower viscosity and obtain better spectral definition. Typical conversion curves are reported in Figures 1a and 1b. It is apparent that BP reacts with L-cystine remarkably faster than BAC. The reduced reactivity of BAC with L-cystine can be ascribed to electrostatic repulsion, since under the reaction conditions both reactants are negatively charged.

The analysis of the experimental data revealed in both cases a pseudo-second-order kinetics, with the kinetic constants including solvent concentration. The best fitting curves, obtained by using eqs 1a and 1b, where [BP], [BAC] and [CYS] represent BP, BAC, and L-cystine molar concentration, respectively, lead to the kinetic constants $k_{c,BP} = 8.10 \times 10^{-3}$ min^{-1} L mol^{-1} for the BP/L-cystine reaction and $k_{c,BAC,} = 1.41 \times 10^{-3}$ min^{-1} L mol^{-1} for for the BAC/L-cystine one.

$$-\frac{d[BP]}{dt} = k_{c,BP}[BP][CYS] \quad (1a)$$

$$-\frac{d[BAC]}{dt} = k_{c,BAC}[BAC][CYS] \quad (1b)$$

No kinetic evidence of multiple addition steps to the same L-cystine molecule was found, confirming that under the conditions adopted L-cystine behaves as a difunctional monomer.

Potentiometric Determination of the Acid Dissociation Constants. The repeating units of BP−CY, having two carboxyl groups and two amino groups, can exist, like parent cystine, in five states of ionization, i.e., LH_4^{2+}, LH_3^+, neutral zwitterionic LH_2, LH^-, and L^{2-}. The repeating units of BAC−CY, featuring one more carboxylic group, can exist in six ionization states, i.e., LH_5^{2+}, LH_4^+, LH_3, LH_2^-, LH^{2-}, and L^{3-}. The corresponding acid dissociation constants K_a, determined by the pH-metric

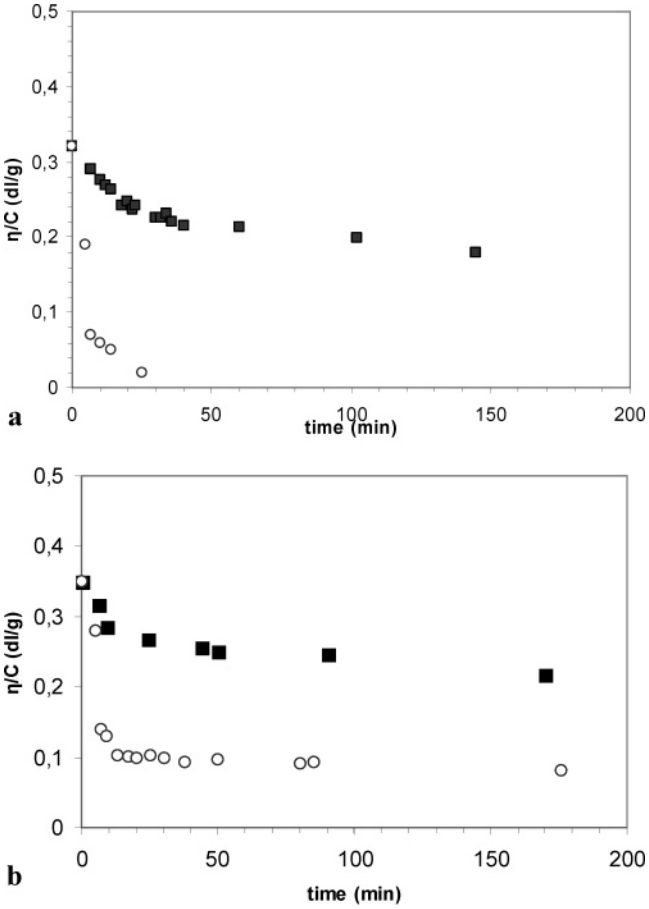


Figure 3. Degradation of BP−CY at 25 °C. Polymer concentration: 4 mg/mL. Panel a: pH 8.3. Panel b: pH 0−1. Key: (■) hydrolytic degradation; (○) reductive degradation in the presence of 1,4-dithio-D,L-threitol.

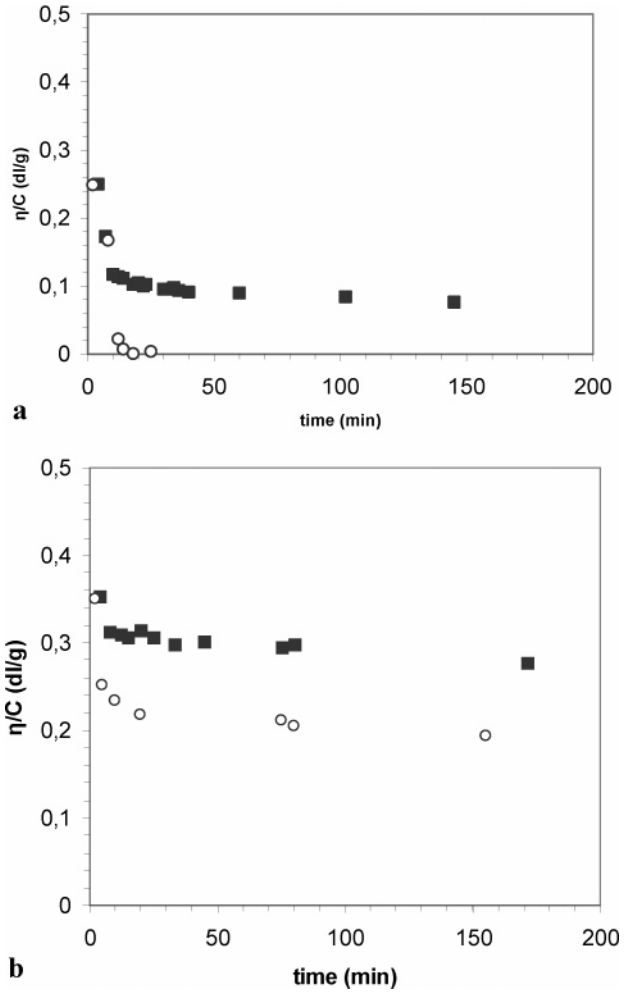


Figure 4. Degradation of BAC−CY at 25 °C. Polymer concentration: 4 mg/mL. Panel a: pH 8.3. Panel b: pH 0−1. Key: (■) hydrolytic degradation; (○) reductive degradation in the presence of 1,4-dithio-(D,L)-threitol.

titration procedure described in the Experimental Part, are reported in Figure 2, contrasting the values obtained from forward and backward titration (a) according to the De Levie modelization[25] and also, when available, (b) from the approximate treatment hinging on the β interaction parameter.[26] The different approaches are in satisfactory agreement. The same figure also reports the β parameters obtained for the two polymers from preliminary analysis of the buffer region, pointing in both cases to moderate but significant interactions between similar neighboring groups. Figure 2 also includes the speciation diagrams for the three compounds in the pH range from 2 to 10. In particular, concerning the physiological pH range, extending from 7.4 in extracellular fluids to 5 in some intracellular compartments, we get that (a) at pH 5, the three compounds are completely in the neutral LH_2 or LH_3 forms; (b) at pH 7.4, the three compounds are mostly (75%-80%) in the neutral LH_2 or LH_3 forms with a significant amount (20−25%) of the negatively charged LH^- or LH_2^- forms which become the prevailing ones at pH ∼ 9.

The four pK_a values obtained for the BP−CY are similar to the parent cystine ones; both compounds exhibit a strongly acid and a weakly to strongly acid carboxylic group, while both the amino groups of BP−CY, in particular the second one, are more basic than the L-cystine ones, which is consistent, inter alia, with the typical basicity sequence of amines (i.e., secondary > primary). The additional carboxylic group on the BAC−CY results much weaker than the other two ones and induces a significant basicity increase on the weaker amine group. This situation resembles the well-known case of ethylenediaminetetraacetic acid (EDTA), whose carboxylic groups have $K_{a1} = 1.02 \times 10^{-2}$, $K_{a2} = 2.14 \times 10^{-3}$, $K_{a3} = 6.92 \times 10^{-7}$, $K_{a4} = 5.50 \times 10^{-11}$, while both its amine groups are so strongly basic that no pK_a value is usually provided for them.

Degradation Studies. The degradation of BP−CY and BAC−CY was followed viscometrically under normal hydrolytic conditions at pH 8.3 and under strongly acid conditions, that is, in the pH range 0−1. At the same pH values the reductive degradation was also investigated in the presence of 1,4-dithio-D,L-treitol as reducing agent. The variation of the reduced viscosity vs time for BP−CY and BAC−CY under different conditions is reported in Figures 3 and 4. It may be observed that the degradation rate is significantly higher at higher pH, but in any case it takes several days to bring down the polymers to oligomeric level.

The degradation of both polymers is dramatically faster in the presence of 1,4-dithio-D,L)-threitol as reducing agent. It may be observed that under basic conditions the molecular weights drop to a very low level in a matter of minutes, making it difficult to determine the initial viscosity values. In acid conditions, the reductive degradation is faster than the hydrolytic one, although the difference is less dramatic than at basic pH values.

Electrochemical Activity Studies. The electrochemical activity of the disulfide bonds in the polymer backbone was

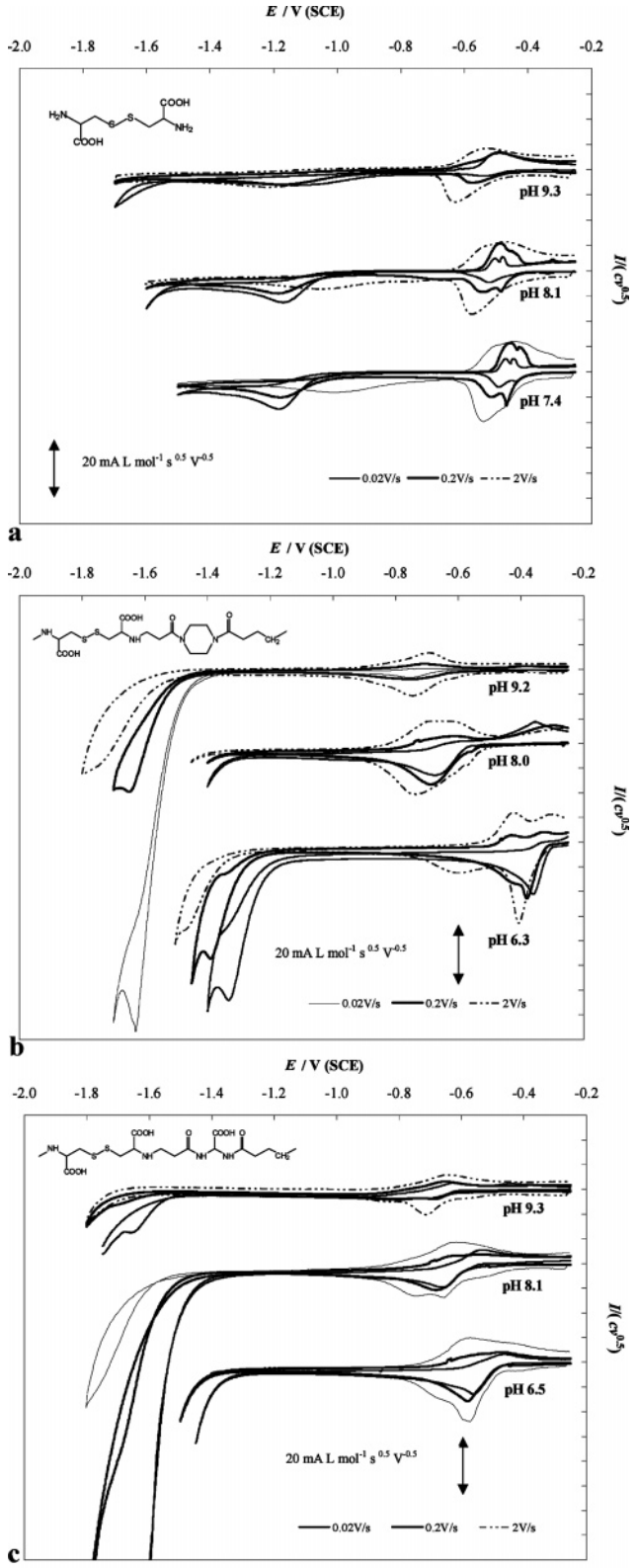


Figure 5. CV patterns on a hanging drop mercury electrode at different pH values and different potential scan rates. Panel a: parent compound L-cystine. Panel b: BP−CY. Panel c: BAC−CY.

investigated together with that of parent L-cystine (as a reference) by cyclic voltammetry on the hanging drop mercury electrode (HDME), in aqueous TRIS-buffered media, at different pH values in the range from 6 to 9.5. A selection of the results is shown in Figures 5a (parent L-cystine), 5b (BP−CY), and 5c (BAC−CY). The CV features obtained for parent L-cystine (Figure 5a) are consistent with previous works in different media by Bard et al.[28] and Heyrovský et al.,[29] since they include the following:

(a) A first cathodic peak system in a potential range between −0.4 and −0.7 V (SCE) is observed, corresponding to reduction of a spontaneously formed thiolate layer,

$$\mathrm{RSSR} = (\mathrm{RSSR})_{ads} \quad (2)$$

$$(\mathrm{RSSR})_{ads} + [\mathrm{HgHg}]_s = [\mathrm{RS{-}HgHg{-}SR}]_s \quad (3)$$

$$[\mathrm{RS{-}HgHg{-}SR}]_s + 2\,e \rightarrow 2\,\mathrm{RS}^- + [\mathrm{HgHg}]_s \quad (4)$$

$$2\mathrm{RS}^- + 2\mathrm{H}^+ = 2\mathrm{RSH} \quad (5)$$

and exhibiting a regular (linear) dependency of the peak potential on pH, which is consistent with the proposed reaction scheme. At the two lower pHs, i.e., when L-cystine is mainly in the globally neutral form (Figure 2) we notice a peak splitting into a sharper, adsorptive one and a second, less sharp one, associated with the structural transition from a more compact thiolate layer (with lateral interactions between the ionizable groups on the L-cystine side chains) to a looser one (with no lateral interactions). Upon increasing the potential scan rate, the two peaks merge, indicating that the above transition is a relatively slow one.

(b) A second cathodic peak, irreversible and of purely diffusive character, corresponding to steps

$$\mathrm{RSSR} + e = [\mathrm{RSSR}]^{\bullet -} \quad (6)$$

$$[\mathrm{RSSR}]^{\bullet -} \rightarrow \mathrm{RS}^- + \mathrm{RS}^{\bullet} \quad (7)$$

$$\mathrm{RS}^{\bullet} + e \rightarrow \mathrm{RS}^- \quad (8)$$

$$2\mathrm{RS}^- + 2\mathrm{H}^+ = 2\mathrm{RSH} \quad (9)$$

i.e. to the direct reduction of L-cystine without any mercury interaction, is then observed. In comparison to the cited works, carried out in acetate or phosphate buffers, this peak is shifted at much more negative potentials (about −1.2 V(SCE) instead of −0.9 V(SCE)); this is consistent with a much higher screening effect from our supporting electrolyte cation (tris(hydroxymethyl) ammmoniummethane).

(c) An anodic peak system follows, nearly symmetrical to the first cathodic peak system, corresponding to oxidative formation of the thiolate layer (from mercury and the cysteine produced in the forward scan), and, at sufficiently low pH and scan rate, to its transition to the more compact structure (made evident by the peak splitting).

Turning to our new L-cystine-based polymers, they feature (Figures 5b and 5c) a first cathodic peak system, similar to the L-cystine one for many aspects, e.g.: (a) it is located at potentials near to the L-cystine one; (b) it is also linearly dependent on the working pH, although with a different (higher) slope (i.e., possibly, a different stoichiometry); (c) it also tends to split, albeit less neatly, into two peaks, the first one (varying linearly with v) being sharper than the second one; (d) in particular, such splitting emerges with decreasing pH and at increasing scan rate.

Therefore, also the polymer first reduction peak system can be associated with the reduction of a spontaneously formed mercurous thiolate layer, as in the case of the parent compound, moreover, also in the polymer case there is some evidence of structural transitions involving side interactions, in spite of the decrease of freedom degrees. However, a significant difference emerges between the polymers and their parent compound when considering the charge of the first reduction peak systems and

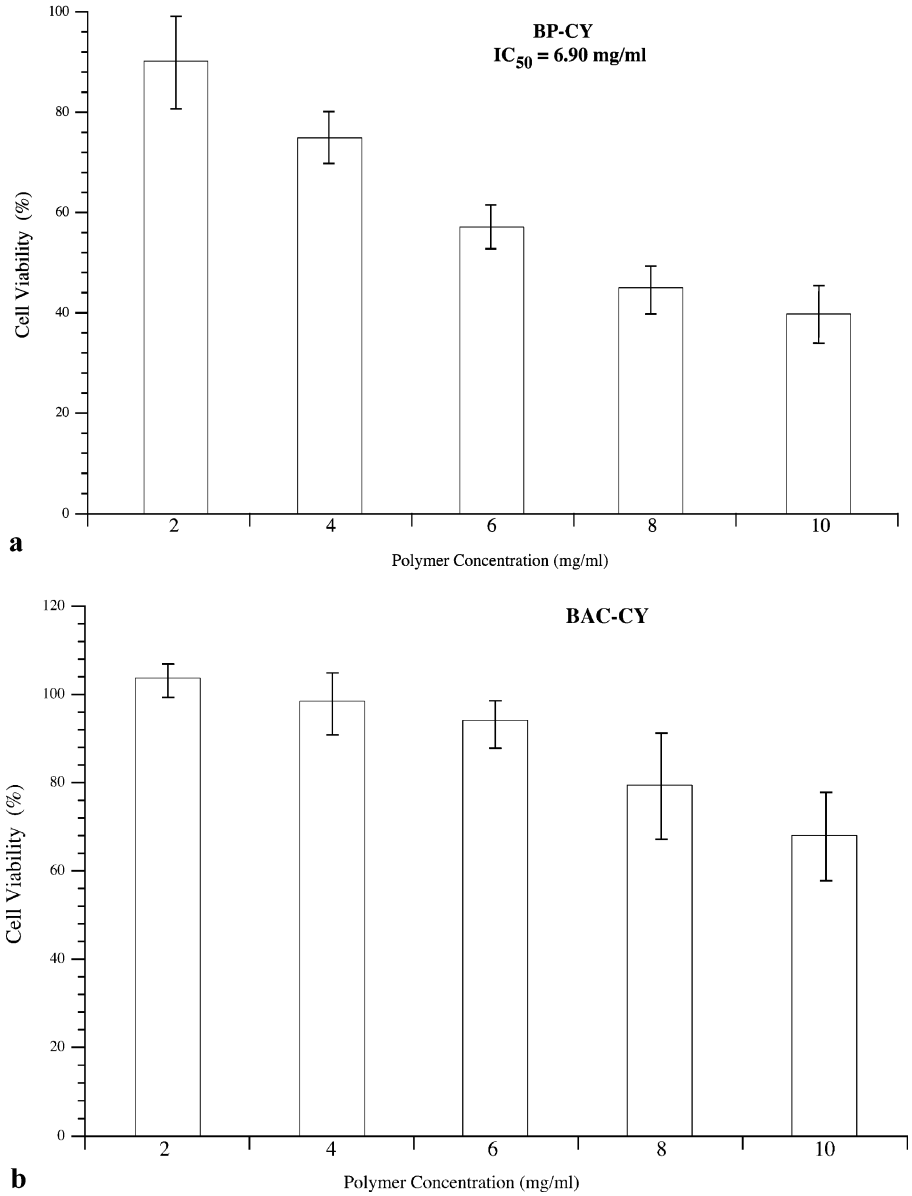


Figure 6. Quantitative results of the cytotoxicity evaluation of L-cystine based PAAs. Panel a: BP−CY. Panel b: BAC−CY.

the dependence of both the peak area and current on the substrate concentration. In particular, for parent compound L-cystine the charges associated with both the sharp structural transition peak and the subsequent desorption peak are fully consistent with the 40−80 μC cm^{-2} specific factors reported in the cited literature,[28] pointing to surface-limited phenomena. Instead, in the polymer cases, the global areas and associated charges are far too high to be associated with a surface process; moreover, they increase linearly with the substrate concentration with no saturation effect up to 0.00075 M at least. Finally, in the polymer cases we observe no second reduction (diffusive) peak in the potential range in which the L-cystine one is located, although a prewave can be observed on the background current, resembling the typical catalytic waves often assigned to hydrogen evolution from the SH group in the literature concerning the electrochemistry of organic sulfur compounds.[29] These observations point to the polymer diffusive reduction peak being merged in the first reduction peak system, with a remarkable anticipation in the corresponding peak potential with respect to the L-cystine case. This would mean that in the polymer case the reduction of the unadsorbed disulfide group occurs as soon as the mercury surface becomes free from the thiolate layer, with no competition from the adsorption of TRIS ammonium cation. A possible explanation, more probable than an enhanced intrinsic reactivity of the disulfide group in the polymers with respect to parent L-cystine, could be that the polymer remains in situ after the thiolate layer reduction, and therefore reacts immediately with no screening effect from adsorbed TRIS cations.

Preliminary Toxicological Evaluations. In vitro evaluation of cytotoxicity is a rapid, standardized, sensitive, and inexpensive way to assess at an early stage the potential biocompatibility of newly synthesized materials. Cell morphology and enzymatic activity are commonly used to investigate the material toxicity. The isolation of test cells in cultures and the absence of the protective mechanisms that assist the cells within the body guarantee for a high sensitivity.[30]

BP−CY and BAC−CY were dissolved in DMEM at different concentrations between 10 and 2 mg/mL. Cells were incubated for 24 h with the polymer solutions and then analyzed for viability with WST-1 tetrazolium salt, which allows for the quantitative evaluation of metabolically active cells. In viable cells, mithocondrial dehydrogenase enzimatically converts the tetrazolium salt WST-1 into soluble formazan, which is quanti-

fied spectrophotometrically at 450 nm and correlates directly with the number of viable cells present in the culture.

Both the investigated polymers resulted highly cytocompatible, even if small differences among the two were noticed. In the case of BP−CY polymer the high value of IC_{50} obtained, which resulted of 6.90 g/L, certainly confirms the extremely good cytocompatibility of the tested compound (Figure 6a). In the case of the polymer BAC−CY no appreciable cytotoxicity was detected even at the higher concentration tested (10 mg/mL), thus it was not possible to determine a value of IC_{50} and the material can be considered fully cytocompatible (Figure 6b).

Conclusions

From the results obtained in this study, a number of relevant conclusions may be drawn:

L-Cystine, a natural α-aminoacid, in spite of carrying four amine hydrogens behaves as a difunctional monomer in Michael-type polyadditions, leading to new linear PAAs, BP−CY and BAC−CY, containing disulfide bonds in the main chain. The polymerization reaction follows pseudo-second-order kinetics.

The molecular architecture of BP−CY and BAC−CY exhibits two distinct features, the presence of *sec*- instead of *tert*-amine groups in the main chain, and a repeating unit formed by two moieties connected by a disulfide linkage. As a consequence, these PAAs degrade by a different mechanism according to the surrounding medium. In aqueous systems containing no reducing agents and at physiological pH, they degrade at approximately the same rate as "traditional" PAAs.

In the presence of reducing agents, both polymers undergo a very fast degradation, no doubt triggered by the reduction of the disulfide bonds.

From the electrochemical standpoint, the disulfide groups in the polymer repeating units appear to behave similarly to free L-cystine, and their electrochemical activity is significantly modulated by the pH. No evidence of a screening effect from the random coil structure was observed; on the contrary, it seems that the interaction of polymer with the mercury surface promotes the bulk reduction of the unadsorbed disulfide groups, which, unlike the parent compound (screened by the adsorbed supporting electrolyte cation), appears to take place immediately after the reductive desorption of the spontaneously formed thiolate layer.

The above experimental facts have at least three major implications.

The first is that drug polymer conjugates can be easily obtained from both BP−CY and BAC−CY owing to the presence of *sec*-amine and carboxyl groups. The conjugates will degrade preferentially inside cells through a reduction mechanism.

Second, if degradation inside cells of a whatever PAA is desired, for instance for PAAs to be employed as transfection agents, this will be easily achieved by introducing L-cystine in the polymerizing mixture, thus leading to L-cystine-containing, and hence degradable, copolymers.

Third, the synthetic results obtained with L-cystine can be probably extended to any peptide α,ω-terminated by α-aminoacidic residues, thus opening the way for novel hybrid PAA-peptide structures in which the peptide moieties are an integral part of the polymer chain, and whose biological properties deserve to be investigated. This will be the object of forthcoming papers.

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References and Notes

(1) Kudo, Y.; Ueshima, H.; Sakai, K. PCT Int. Appl. WO 0074720, December 14, 2000.
(2) Schacht, E.; Wilding, I. PCT Int. Appl. WO 9610994, April 18, 1996.
(3) Woghiren, C.; Sharma, B.; Stein, S. *Bioconjugate Chem.* **1993**, *4*, 314.
(4) Huang, S.; Pooyan, S.; Wang, J.; Choudhury, I.; Leibowitz, M.; Stein, S. *Bioconjugate Chem.* **1998**, *9*, 612.
(5) Zalipsky, S.; Qazen, M.; Walker, J. A.; Mullah, N.; Quinn, Y. P.; Huang, S. K. *Bioconjugate Chem.* **1999**, *10*, 703.
(6) Ishida, T.; Kirchmeier, M. J.; Moasea, E. H.; Zalipsky, S.; Allen, T. M. *Biochim. Biophys. Acta* **2001**, *1515*, 144.
(7) Zalipsky, S.; Gabizon, A. A. PCT Int. Appl. WO 2000064484, November 11, 2000.
(8) Danusso, F.; Ferruti, P. *Polymer* **1970**, *11*, 88.
(9) Ferruti, P.; Marchisio, M. A.; Barbucci, R. *Polymer* **1985**, *26*, 1336.
(10) Ferruti, P. Ion-Chelating Polymers (Medical Applications). In *Polymeric Materials Encyclopedia*; Salamone, J. C., Ed.; CRC Press Inc.: Boca Raton, FL, 1996; Vol. 5, p 3334−3359.
(11) Ferruti, P.; Marchisio, M. A.; Duncan, R. *Macromol. Rapid Commun.* **2002**, *23*, 332.
(12) Franchini, J.; Ferruti, P. *J. Bioactive Compat. Polym.* **2004**, *19*, 221.
(13) Ferruti, P.; Ranucci, E.; Sartore, L.; Bignotti, F.; Marchisio, M. A.; Bianciardi, P.; Veronese, F. M. *Biomaterials* **1994**, *15*, 1235.
(14) Ferruti, P.; Ranucci, E.; Bignotti, F.; Sartore, L.; Bianciardi, P.; Marchisio, M. A. *J. Biomater. Sci. Polym. Ed.* **1994**, *6*, 833.
(15) Barbucci, R.; Casolaro, M.; Ferruti, P.; Barone, V.; Lelj, F.; Oliva, L. *Macromolecules* **1981**, *14*, 1203.
(16) Ranucci, E.; Spagnoli, G.; Ferruti, P.; Sgouras, D.; Duncan, R. *J. Biomater. Sci. Polym. Ed.* **1991**, *2*, 303.
(17) Richardson, S.; Ferruti, P.; Duncan, R. *J. Drug Targeting* **1999**, *6*, 391.
(18) Richardson, S.; Man, S.; Ferruti, P.; Manzoni, S.; Duncan, R. *Proceedings of the International Symposium on Controlled Release of Bioactive Materials*, Boston, MA, 1999; Vol. 26, pp 165−166.
(19) Emilitri, E.; Ranucci, E.; Ferruti, P. *J. Polym. Sci., Part A: Polym. Chem.* **2005**, *43*, 1404.
(20) Ferruti, P.; Knobloch, S.; Ranucci, E.; Duncan, R.; Gianasi, E. *Macromol. Chem. Phys.* **1998**, *199*, 2565.
(21) Ferruti, P.; Ranucci, E.; Trotta, F.; Gianasi, E.; Evagorou, G. E.; Wasil, M.; Wilson, G.; Duncan, R. *Macromol. Chem. Phys.* **1999**, *200*, 1644.
(22) Ferruti, P. *Macromol. Synth.* **1985**, *9*, 25.
(23) Hurd, R. E.; John, B. K. *J. Magn. Reson.* **1991**, *91*, 648.
(24) Ruiz-Cabello, J.; Vuister, G. W.; Moonen, C. T. W.; van Gelder, P.; Cohen, J. S.; van Zijl, P. C. M. *J. Magn. Reson.* **1992**, *100*, 282.
(25) De Levie, R. *Aqueous Acid-Base Equilibria and Titrations*; Oxford University Press: Oxford, U.K., 1999.
(26) Katchalsky, A.; Spitnik, P. *J. Polym. Sci.* **1947**, *2*, 432.
(27) Ranucci, E.; Macchi, L.; Annunziata, R.; Ferruti, P.; Chiellini, F. *Macromol. Biosci.* **2004**, *4*, 706.
(28) Stankovich, M. T.; Bard, A. J. *J. Electroanal. Chem.* **1977**, *75*, 487.
(29) Heyrovský, M.; Mader, P.; Veselá, V.; Fedurco, M. *J. Electroanal. Chem.* **1994**, *369*, 53 and literature cited therein.
(30) ISO 10993 [EN 30993] Part 10995: Test for cytotoxicity—*in vitro* methods.

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