Functionalization and Molecular Dynamics Study of Carboxy-Terminated Poly(1-vinylpyrrolidin-2-one): A Potential Soluble Carrier of Biomolecules

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ABSTRACT: The reactivity of carboxy-terminated poly(1-vinylpyrrolidin-2-one) (PVP-COOH) 40-mers **1** with various small bi-functionalized molecules has been investigated. A number of new differently functionalized PVP **3–11** have been successfully obtained demonstrating that the presence of the bulky PVP chain did not hamper the reactivity of the carboxy group. This would imply that in solution the carboxyl group is not buried inside the coil, but well exposed to the solvent, as further confirmed by a molecular dynamics conformational study. © 2008 Wiley Periodicals, Inc. J Polym Sci Part A: Polym Chem 46: 1683–1698, 2008

Keywords: computer modeling; functionalization of polymers; MALDI; molecular dynamics; PVP; water-soluble polymers

INTRODUCTION

The covalent attachment of drugs to water-soluble polymers[1–3] is an expanding area of pharmaceutical research, as the polymer conjugation of bioactive molecules is a promising strategy for improving their pharmacological profile and therapeutic index.[4] A number of soluble polymers having linear, hyperbranched, or dendritic structures have been tested for the conjugation of low- and high-molecular weight bioactive molecules, giving rise to the so-called polymer therapeutics.[5–7] For example, PEG conjugation of therapeutically relevant proteins is a well-established technology, and a number of PEG-ylated compounds have received market approval or are in advanced clinical trials.[8–12]

Poly(1-vinylpyrrolidin-2-one) (PVP) is another well-known biocompatible soluble polymer widely used in many different applications.[13–17] It might be a promising candidate for polymer therapeutics.[18,19] PVP has been used as plasma expander,[20] but is not biodegradable. Because of problems in metabolism and excretion of the high-molecular weight fractions, that use, as any other use involving parenteral administration, was later abandoned. Only low-molecular weight PVP oligomers can be bioeliminated by renal filtration, up to a M_w of ~40,000 D.

Leaving apart any bioelimination problems, the absence of reactive functional groups along

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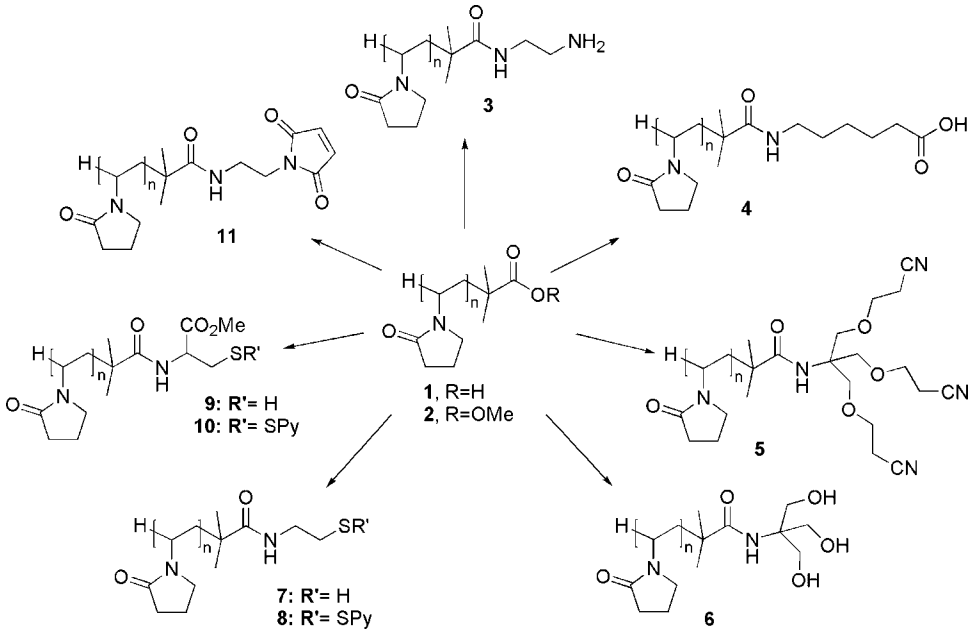


Scheme 1. New end-functionalized derivatives obtained from PVP-COOH **1**.

the polymer chain prevents the extensive use of PVP for covalent drug conjugation,[21] thus making its functionalization a relevant synthetic target in polymer chemistry. The conventional method for preparing functionalized PVP is by copolymerization of 1-vinylpyrrolidin-2-one with suitable acrylic monomers. However, this copolymerization is not straightforward, because of large differences in reactivity ratios, leading to compositional drift with yield, which results in uneven distribution of functional units.[22] Some examples of lactam ring-functionalized PVP useful as precursors of graft copolymers have been proposed for this purpose with better results.[23–27] All these derivatives are not biodegradable, and this limits their potential as biomedical polymers.

For applications not requiring high-molecular weight polymers, the most convenient method for obtaining bioeliminable functionalized PVP is the radical polymerization of 1-vinylpyrrolidin-2-one in the presence of functionalized chain transfer agents. This allows the direct preparation of low-molecular weight end-functionalized PVP,[28–33] useful for the covalent modification of biomolecules.[34–38] Extending the number and the nature of PVP end-groups certainly deserves additional search.

To this purpose we investigated the reactivity of carboxyl group of PVP **1** (PVP-COOH) toward a series of bi-functionalized molecules for obtaining different new functionalized polymers. PVP **1** was chosen as suitable starting polymer, because its synthesis and characterization is well defined[31] and the carboxyl group is a versatile function from a synthetic point of view.

In this article, we report an investigation of the reactivity of PVP-COOH **1**, from which a series of new end-functionalized PVP derivatives **3–11** have been prepared (Scheme 1).

Some of them were also tested for the conjugation of a bioactive small molecule, such as reduced glutathione. The experimental results were further integrated and supported by a molecular modeling investigation of **1** in aqueous solution. In fact, in spite of its huge number of applications, just one work dealing with a study of molecular modeling of PVP has been published,[39] but nothing has been reported on the conformational studies of end-functionalized PVP polymers.

EXPERIMENTAL

Materials and Methods

Analytical grade solvents and reagents were purchased from Fluka or Aldrich and used as received, unless otherwise stated. All operations were performed under nitrogen atmosphere and

using dried glassware. 1-Vinylpirrolidin-2-one (VP) (97%) was distilled before use; 2,2′-Azobis(2-methyl propionitrile) (AIBN) (98%) was crystallized from isopropanol. The buffer solution, pH = 8, was prepared adding 0.6 g of TRIZMA® [tris(hydroxymethyl)aminomethane] to a 2.9 mL of HCl 0.1 M solution, water was added to 50 mL, and the solution was eventually deoxygenated by bubbling N_2 under ultrasounds.

Amicon stirred ultrafiltration cell, mod. 8050, was used for the purification of the polymers.

Abbreviations: *N*,*N*′-dicyclohexylcarbodiimide (DCC); *N*,*N*-diisopropylethylamine (DIEA); *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate (HBTU); *N*,*N*-dimethyl formamide (DMF); tertbutoxycarbonyl (Boc); carbobenzyloxy (Cbz); 1,4-Dithio-DL-threitol (DTT); Pyridine (Py); Pyrrolidinone (Pyr).

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC300, AMX 300, and Avance 500 MHz spectrometer. IR spectra were recorded on a Perkin-Elmer 1725 FTIR spectrometer. MALDI-TOF spectra were obtained on a Bruker OMNIFLEX instrument with a linear time-of-flight analyzer. Ions formed by a pulsed UV laser beam (nitrogen laser, 337 nm) were accelerated through 19 kV. Reported mass spectra are the results of 150–250 laser shots. The spectrometer calibration was performed using a mixture of peptides with molecular weight below 20,000 purchased from Bruker. Matrix solution was prepared dissolving α-cyano-4-hydroxycinnamic acid (CHCA) in H_2O/CH_3CN 80:20 mixture. The sample was prepared dissolving the polymer (1 mg) in water (300 μL) (HPLC grade) and mixing with the matrix solution in 2/1 v/v ratio, 1 μL of such solution was cast on the stainless scout probe and dried at room temperature ("dried droplet" technique).

Size exclusion chromatography (SEC) traces and molecular weight determinations were obtained by an equipment obtained by assembling a Knauer Pump 1000 with Knauer Autosampler 3800 two TosoHaas columns (TSK-gel G4000 PW and G3000 PW) connected in series, and a refractive index detector Waters model 2410. Mobile phase: TRIZMA® pH = 8.0, 0.1 M solution + NaCl 0.2 M solution; flow rate: 1 mL/min. The samples were added as 1% solution in the same buffer. Molecular weight determinations were based on a calibration curve obtained with PEG standards.

MALDI-TOF End-Group Analysis

MALDI-TOF spectra obtained in this way for most of the PVP derivatives lead to following general observations: (see for instance Chart 12 of compound 24).

a. All spectra exhibited a major distribution and several minor ones ascribable to the sodium or potassium cationization of PVP macromolecules. All the molecular species present in the samples were terminated with the expected function. No distribution ascribable to the starting PVP-COOH **1** was present.
b. The mass difference of the adjacent peaks of both major and minor subdistributions were invariably equal to the molecular mass of VP repeating unit.
c. The measured masses of the component polymers in this molecular weight distribution can be expressed by the following equation.[40,41]

$$M = n \times M_{\mathrm{mon}} + M_{\mathrm{end}} + M_{\mathrm{cat}}$$

where n = degree of polymerization; M = macromolecule mass; M_{mon} = repeating unit mass (VP = 111.07); M_{end} = end-group mass; M_{cat} = cation mass (H^+; Na^+, K^+); thus straight line equation can be written as: $y = 111.07x + (M_{\mathrm{end}} + M_{\mathrm{cat}})$.

The measured molecular mass of the polymer molecules can be plotted against the degree of polymerization, the slope of the fitted line gives the mass of the repeating unit, and the intercept with y axis represents the mass of the end group and the cation.

For the sake of comparison we plotted in a graphic the measured and calculated M values for most of the obtained end-terminated polymers. Dashed line: calculated M; bold dashed line: experimental values. A very good agreement between theoretical and experimental results were found in all cases.

Computational Methods

Molecular dynamic simulations and analysis were performed using the Amber9 program package,[42] with the General Amber Force Field.[43] Missing parameters for pyrrolidone were derived by HF/6-31G* calculations con-

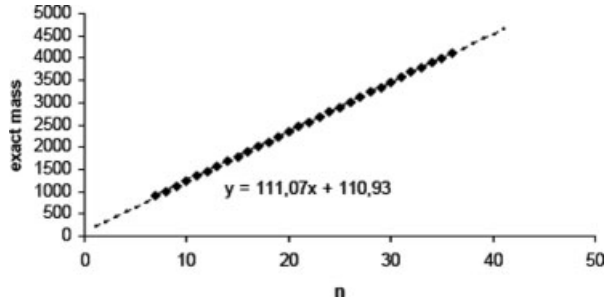


Chart 1. Calc.: 111.07x + 88.05; Exp.: 111.07x + 110.93 (87.94 + Na) (error ~ 1%).

duced on atactic, sindiotactic, and isotactic polymers consisting of four vinyl pyrrolidone monomers. *Ab initio* calculations were performed with the Gaussian03 programme package.[44] Final bond length and angle parameters were derived by a least square fitting of all the analogue bonds and angles obtained from the *ab initio* geometries. The RED program was used to calculate accurate RESP point charges,[45] and a set of two different spatial conformation for each PVP tetramer was used for charge averaging. The solvent was treated by mean of the GB implicit solvation model for water.[46] Production runs were performed by using an infinite cut-off for electrostatic and a time step of 0.002 ps. The SHAKE algorithm was used to constrain all bonds involving hydrogens.[47] The simulation was performed at constant temperature by using the weak-coupling algorithm.[48]

PVP-COOH **1** was synthesized following the literature procedure,[28–31] and was dried, before use, by heating for 5 h at 60 °C under vacuum. $\overline{M}_n$ = 4535 by titration; $\overline{M}_n$ = 3001, $\overline{M}_w$ = 5247, PD = 1.748 by SEC analysis.

MALDI-TOF end-group analysis of PVP-COOH **1** (Chart 1).

General Procedure for the Isolation of the Polymers (200 mg Scale)

The following procedure was used to isolate PVP derivatives (unless otherwise described). At the end of the reaction, the residue from the solvent evaporation, was taken up in CH_2Cl_2 (2 mL) and the solution slowly dropped under stirring to Et_2O (100 mL). The solvent was decanted and the precipitate, after drying over reduced pressure, was dissolved in deionized water (50 mL) then purified by ultra filtration (4 × 40 mL H_2O) through a membrane with a nominal cut off of 3000. The polymer was isolated as a powder after lyophilization.

PVP *N*-(2-Boc-aminoethyl) carboxamide 12a

HBTU (122 mg, 0.32 mmol) and DIEA (0.1 mL) were added to a solution of PVP-COOH **1** (290 mg, 0.064 mmol) in dry DMF (2 mL). The mixture was stirred for 5 min at room temperature then a solution of *N*-*tert*-butoxycarbonyl ethylendiamine (60 mg, 0.32 mmol) in DMF (1 mL) was added.

The reaction was stirred for 3 days at room temperature, then the white polymer **12a** isolated.

^{1}H NMR ($CDCl_3$) δ (ppm) = 1.42–1.68 (m, CH_2 + CH_3); 2.01 (m, CH_2 pyr); 2.24–2.4 (m, CH_2CO); 3.22 (m, CH_2N + CH_2), 3.73–4.0 (m, CH). ^{13}C NMR ($CDCl_3$) δ (ppm) = 18.4, 31.7, 35.7, 42.3, 45.0.

MALDI-TOF end-group analysis of **12a** (Chart 2).

PVP-(2-Cbz-aminoethyl) carboxamide 12b

HBTU (88 mg, 0.22 mmol) and DIEA (0.07 mL) were added to solution of PVP-COOH **1** (200 mg, 0.044 mmol) in dry DMF (2 mL). The mixture was stirred for 5 min at room temperature then a solution of *N*-benzyloxycarbonyl ethylendiamine (43 mg, 0.22 mmol) in DMF (1 mL) was added. The reaction was stirred for 3 days at room temperature, and the polymer **12b** was isolated.

^{1}H NMR ($CDCl_3$) δ (ppm) = 1.44–1.71 (m, CH_2 + CH_3); 2.05 (m, CH_2 pyr); 2.25–2.38 (m, CH_2CO); 3.24 (m, CH_2N + CH_2); 3.65–3.9 (m, CH); 5.13 (m, —OCH_2Ph); 7.36 (m, Ph). ^{13}C NMR ($CDCl_3$) δ (ppm) = 18.3, 31.7, 35.3, 42.0, 45.2, 69.6, 128.0–128.4.

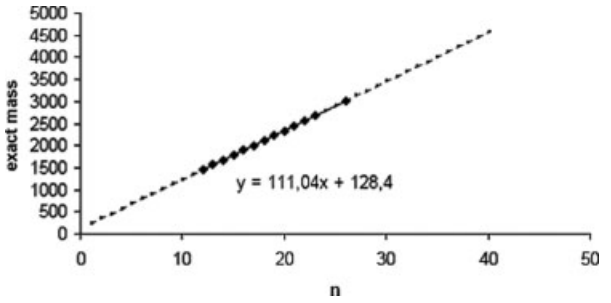


Chart 2. Calc.: 111.07x + 130.11; Exp.: 111.04x + 128.4 (error ~ 1.3%).

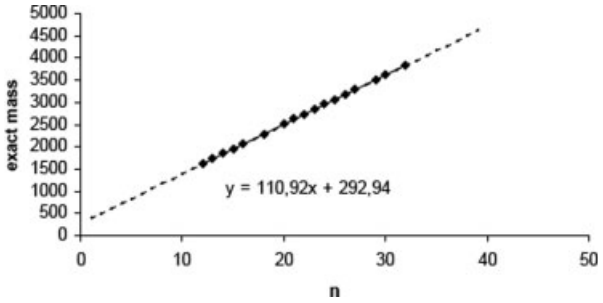


Chart 3. Calc.: 111.07x + 264.15; Exp.: 110.92x + 292.94 [+Na] (error ~ 2%).

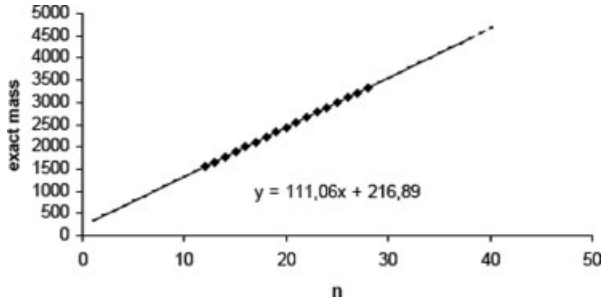


Chart 4. Calc.: 111.07x + 215.15; Exp.: 111.06x + 216.89 (error ~ 1%).

MALDI-TOF end-group analysis of **12b** (Chart 3).

PVP (2-aminoethyl) carboxamide 3

From 12a by Boc Cleavage

Trifluoroacetic acid (0.03 mL, 0.5 mmol) was added, at room temperature, to a solution of **12a** (150 mg, 0.033 mmol) in CH_2Cl_2 (1 mL) and the reaction was stirred for 2 days at the same temperature. The solvent was evaporated, the residue was dissolved in water (50 mL), and directly ultrafiltered to give **3** as trifluoroacetate salt.

^{1}H NMR ($CDCl_3$) δ (ppm) = 1.41–1.68 (m, CH_2 + CH_3); 1.99–2.02 (m, CH_2 pyr); 2.22–2.35 (m, CH_2CO); 3.21 (m, CH_2N + CH_2); 3.70–3.89 (m, CH).^{13}C NMR ($CDCl_3$) δ (ppm) = 18.2, 31.1, 35.3, 42.0, 45.2. ^{19}F NMR ($CDCl_3$) δ (ppm) = −76.23 (s, CF_3).

From 12b by Cbz Clevage

Pd/C (10%) (100 mg) and $HCOONH_4$ (50 mg, 0.49 mmol) were added to a solution of **12b** (460 mg, 0.098 mmol) in MeOH (4 mL); the reaction was stirred for 3 days at room temperature and then filtered over a pad of celite®. The solvent was evaporated, the residue was dissolved in water (50 mL), and directly ultrafiltered to give **3**.

^{1}H NMR ($CDCl_3$) δ (ppm) = 1.42–1.7 (m, CH_2 + CH_3); 2.04 (m, CH_2 pyr) 2.24–2.35 (m, CH_2CO); 3.22 (m, CH_2N + CH_2); 3.72–3.87 (m, CH). ^{13}C NMR ($CDCl_3$) δ (ppm) = 18.2, 31.2, 35.4, 42.4, 45.2.

PVP-aminohexanoic acid methyl ester 14

HBTU (120 mg, 0.31 mmol) and DIEA (0.1 mL) were added to a solution of PVP-COOH **1** (240 mg, 0.053 mmol) in dry DMF (2 mL). The mixture was stirred for 5 min at room temperature then a solution of 6-amino methyl hexanoate hydrochloride **13** (60 mg, 0.32 mmol) in DMF (1 mL) was added. The reaction was stirred for 3 days at room temperature. Polymer **14** was isolated as a slight yellow powder.

^{1}H NMR ($CDCl_3$) δ (ppm) = 1.41–1.68 (m, CH_2 + CH_3); 2.04 (m, CH_2 pyr) 2.24–2.34 (m, CH_2CO); 3.20 (m, CH_2N+ CH_2); 3.60–3.9 (m, CH +OCH_3). ^{13}C NMR ($CDCl_3$) δ (ppm) = 18.0, 31.3, 35.7, 42.0, 45.2.

MALDI-TOF end-group analysis of **14** (Chart 4).

PVP-aminohexanoic acid 4

PVP-methylester **14** (180 mg, 0.04 mmol) was dissolved in NaOH (0.1 M, 10 mL). The reaction was stirred at room temperature for 3 days, then HCl 1 M was added up to pH 2.5. The reaction mixture was directly purified by ultrafiltration then polymer **4** isolated as a slight yellow powder.

^{1}H NMR ($CDCl_3$) δ (ppm) = 1.42–1.7 (m, CH_2 + CH_3); 2.04 (m, CH_2 pyr) 2.23–2.37 (m, CH_2CO); 3.22 (m, CH_2N + CH_2); 3.71–3.9 (m, CH). ^{13}C NMR ($CDCl_3$) δ (ppm) = 18.0, 31.3, 35.7, 42.0, 45.2.

MALDI-TOF end-group analysis of **4** (Chart 5).

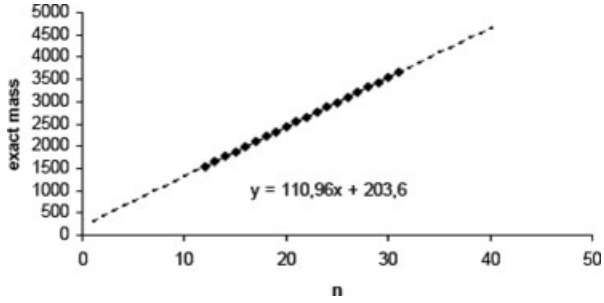


Chart 5. Calc.: 111.07x + 201.13; Exp.: 110.96x + 203.60 (error ~ 2%).

PVP-tris-cyano Derivative 5

Route a: HBTU (90 mg, 0.21 mmol) and DIEA (0.08 mL) were added to solution of PVP-COOH **1** (200 mg, 0.044 mmol) in dry DMF (2 mL). The mixture was stirred for 5 min at room temperature then a solution of tris[(cyanoethoxy)methyl]aminomethane (**15**)[49,50] (95 mg, 0.22 mmol) in DMF (1 mL) was added. The reaction was stirred for 3 days at room temperature shielded from light, then the solvent was evaporated. Polymer 5 was obtained as a slightly yellow powder after lyophilizaton.

Route b: 0.1 mL of KOH (40% H_2O solution) was added to a solution of polymer **6** (60mg, 0.013 mmol) in DMF (2 mL). The red clear solution was stirred at room temperature for 10 min then acrylonitrile (11 mL, 0.2 mmol) was added affording a dark brown solution. The mixture was stirred at room temperature for 3 days. Polymer **5** was isolated as a light brown-yellow powder.

^{1}H NMR ($CDCl_3$) δ (ppm) = 1.41–1.64 (m, CH_2 + CH_3); 2.04 (m, CH_2 pyr) 2.20–2.32 (m, CH_2CO); 3.22–3.5 (m, CH_2N + CH_2O); 3.7–4.2 (m, CH + CH_2CN). ^{13}C NMR ($CDCl_3$) δ (ppm) = 18.2, 31.5, 35.7, 42.1, 45.1. FTIR ($CHCl_3$) ν (cm^{-1}) = 2253 [CN].

PVP-tris-hydroxy Derivative 6

HBTU (132 mg, 0.32 mmol) and DIEA (0.11 mL, 0.7 mmol) were added to a solution of PVP-COOH **1** (300 mg, 0.066 mmol) in dry DMF (3 mL). The mixture was stirred for 5 min at room temperature then a solution of tris(hydroxymethyl)aminomethane (**16**) (50 mg, 0.33 mmol) in DMF (3 mL) was added. The reaction was stirred for 3 days at room temperature. Polymer **6** was obtained as a slight yellow powder.

^{1}H NMR ($CDCl_3$) δ (ppm) = 1.41–1.65 (m, CH_2 + CH_3); 2.03 (m, CH_2 pyr) 2.22–2.32 (m, CH_2CO); 3.24 (m, CH_2N); 3.74–3.89 (m, CH + CH_2). ^{13}C NMR ($CDCl_3$) δ (ppm) = 18.0, 31.6, 35.4, 41.9, 45.3.

MALDI-TOF end-group analysis of **6** (Chart 6).

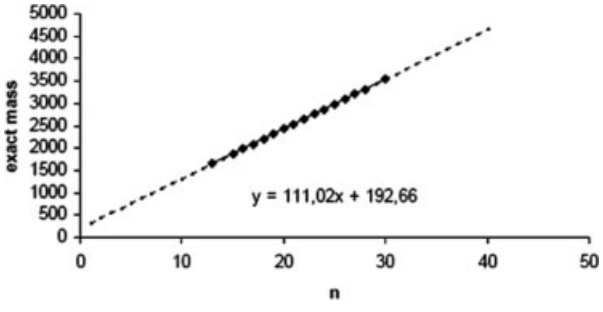


Chart 6. Calc.: 111.07x + 191.11; Exp.: 110.92x + 192.66 (error ~ 1%).

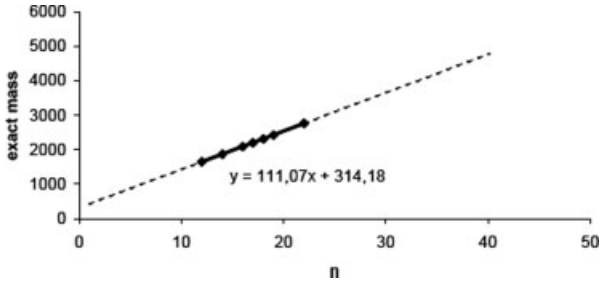


Chart 7. Calc.: 111.07x + 322.14; Exp.: 111.0x + 314.18 (error ~ 2%).

PVP-*N*-Boc cystamine Derivative 18

A slurry of *N*-Boc-cystamine hydrochloride (**17**) (130 mg, 0.4 mmol) and Et_3N (0.05 mL, 0.4 mmol) in dry $CHCl_3$ (2 mL) was added at room temperature to a stirred solution of PVP-COOH **1** (300 mg, 0.066 mmol) in dry $CHCl_3$ (2 mL), after 5 min DCC (130 mg, 0.4 mmol) was added and the yellowish suspension was stirred at room temperature for 3 days. Polymer **18** was obtained as a white powder.

^{1}H NMR ($CDCl_3$) δ (ppm) = 1.41–1.69 (m, CH_2 + CH_3); 2.02 (m, CH_2 pyr); 2.22–2.35 (m, CH_2CO + CH_2); 3.22 (m, CH_2N); 3.70–3.87 (m, CH). ^{13}C NMR ($CDCl_3$) δ (ppm) = 18.2, 31.2, 35.6, 42.0, 45.0.

MALDI-TOF end-group analysis of **18** (Chart 7).

PVP-2-mercaptoethyl carboxamide 7

1,4-Dithio-DL-threitol (DTT) (60 mg, 0.3 mmol) was added to a solution of **18** (110 mg, 0.03 mmol) in TRIZMA®HCl buffer solution pH = 8 (4 mL). The yellowish solution was stirred at room temperature for 3 days then directly purified by ultrafiltration using deoxygenated water. SH-terminated polymer **7** was recovered as a light grey powder and was directly used in the next step.

PVP-dithiopyridyl Derivative 8

A solution of 2,2′-dithiopyridine (60 mg, 0.2 mmol) in deoxygenated EtOH (1 mL) was added to a solution of **7** (80 mg, 0.02 mmol) in deoxygenated EtOH (2 mL). The pale yellow solution

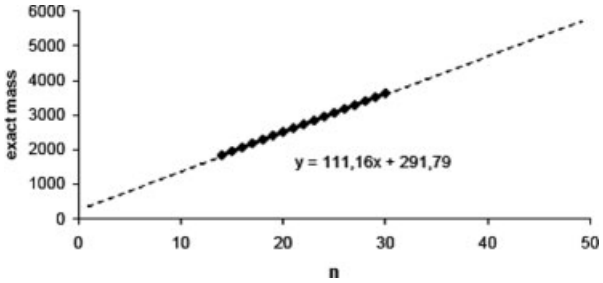


Chart 8. Calc.: 111.07x + 256.07; Exp.:111.16x + 291.79 (error ~ 1.3%).

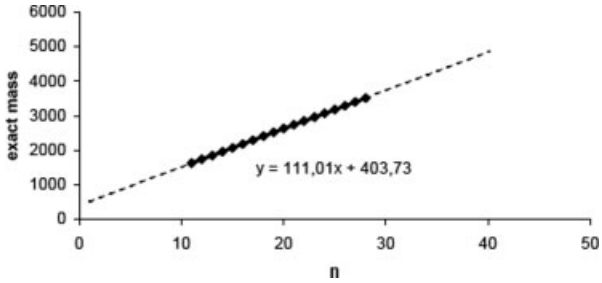


Chart 9. Calc.: 111.07x + 403.19; Exp.: 111.01x + 403.73 (error ~ 1%).

was stirred for 2 days at room temperature, then the mixture was diluted with water (40 mL). A 0.3 M solution of $KHSO_4$ (1 mL) was added to the white slurry giving a clear and yellowish solution (pH = 2). Ethanol was evaporated and the solution was directly purified by ultrafiltration to give **8**.

^{1}H NMR ($CDCl_3$) δ (ppm) = 1.40–1.69 (m, CH_2 + CH_3); 2.02 (m, CH_2 pyr); 2.35 (m, CH_2CO + CH_2S); 3.21 (m, CH_2N); 3.71–3.87 (m, CH); 7.1 (m, CH py); 7.59 (m, CH py); 8.87 (m, CH py). ^{13}C NMR ($CDCl_3$) δ (ppm) = 18.2, 31.3, 34.6, 42.0, 43.6, 44.8, 119.7, 121.1, 137.3, 149.5, 175.4.

MALDI-TOF end-group analysis of **8** (Chart 8).

Bis-PVP-cystamine Derivative 20

HBTU (140 mg, 0.33 mmol) and DIEA (0.3 mL, 2 mmol) were added to solution of PVP-COOH **1** (260 mg, 0.06 mmol) in dry DMF (2 mL). The mixture was stirred for 5 min at room temperature then a solution of cysteamine dihydrochloride (**19**) (150 mg, 0.7 mmol) in DMF (1 mL) was added. The reaction was stirred for 3 days at room temperature. Polymer **20** was obtained as a slight yellow powder.

^{1}H NMR ($CDCl_3$) δ (ppm) = 1.39–1.68 (m, CH_2 + CH_3); 2.02 (m, CH_2 pyr); 2.25–2.33 (m, CH_2CO + CH_2S); 3.22 (m, 2H, CH_2N); 3.69–3.86 (m, CH). ^{13}C NMR ($CDCl_3$) δ (ppm) = 18.2, 31.2, 34.9, 42.5, 44.8.

MALDI-TOF end-group analysis of **20** (Chart 9).

DTT Reduction of 20 to give 7

PVP **20** (260 mg, 0.03 mmol) was then dissolved in TRIZMA® HCl buffer solution pH = 8 (4 mL) and treated with DTT (50 mg, 0.3 mmol). The yellowish solution was stirred at room temperature for 3 days, then directly purified by ultrafiltration using deoxygenated water. SH-terminated polymer **7** was recovered as grey powder.

MALDI-TOF end-group analysis of **7** (Chart 10).

Bis-PVP-cystine Derivative 22

HBTU (125 mg, 0.28 mmol) and DIEA (0.5 mL) were added to a solution of PVP-COOH **1** (220 mg, 0.058 mmol) in dry DMF (3 mL). The mixture was stirred for 5 min at room temperature then **21** (200 mg, 0.58 mmol) was added to the solution, that was stirred for 3 days at the same temperature. Polymer **22** was obtained as a slight yellow powder.

^{1}H NMR ($CDCl_3$) δ (ppm) = 1.38–1.68 (m, CH_2 + CH_3); 2.04 (m, CH_2 pyr); 2.30–2.36 (m, CH_2CO + CH_2S); 3.22 (m, 2H, CH_2N); 3.69–3.86 (m, CH + OCH_3). ^{13}C NMR ($CDCl_3$) δ (ppm) = 18.1, 31.5, 35.2, 43.0, 45.2.

MALDI-TOF end-group analysis of **22** (Chart 11).

PVP-cysteine Derivative 9

DTT (20 mg, 0.1 mmol) was added to a solution of **22** (80 mg, 0.01 mmol) in fresh prepared TRIS buffer solution (4 mL), carefully deoxygenated by bubbling nitrogen under ultrasounds. The solution was stirred at room temperature for

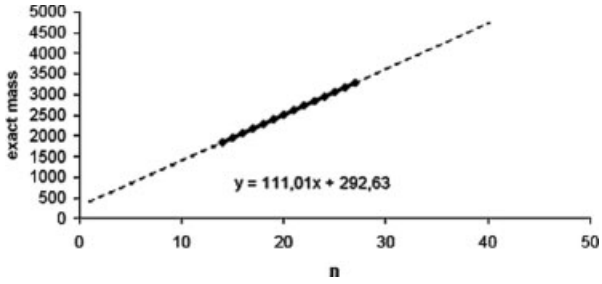


Chart 10. Calc.: y = 111.07x + 292.13; Exp.: y = 111.01x + 292.63 (error ~ 1%).

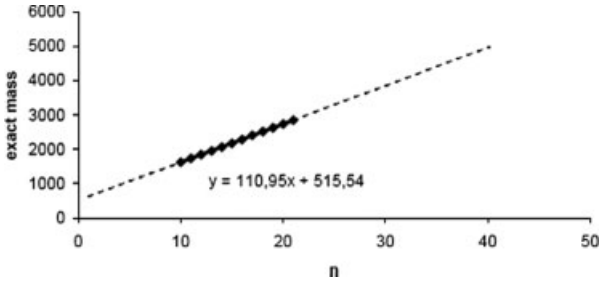


Chart 11. Calc.: 111.07x + 519.21; Exp.: 110.95x + 515.54 (error ~ 1%).

3 days, then was directly purified by ultrafiltration using deoxygenated water. Polymer **9** was isolated as slight yellow powder and directly used in the next step.

PVP-thiopyridylcysteine Derivative 10

A solution of 2,2′-dithiopyridine (55 mg, 0.2 mmol) in deoxygenated EtOH (1 mL) was added to a solution of **9** (80 mg, 0.01 mmol) in deoxygenated EtOH (2 mL). The pale yellow solution was stirred for 2 days at room temperature, then the mixture was diluted with water (40 mL). A 0.3 M solution of $KHSO_4$ (1 mL) was added to the white slurry, giving a clear yellowish solution (pH = 2), which was directly purified by ultrafiltration. Polymer **10** was recovered as a white powder.

^{1}H NMR ($CDCl_3$) δ (ppm) = 1.39–1.69 (m, CH_2 + CH_3); 2.01 (m, CH_2 pyr); 2.33 (m, CH_2CO + CH_2S); 3.20 (m, CH_2N); 3.69–3.88 (m, CH + OCH_3); 7.10 (m, CH py); 7.60 (m, CH py); 8.44 (m, CH py). ^{13}C NMR ($CDCl_3$) δ (ppm) = 18.2, 31.5, 34.2, 42.0, 43.6, 44.8, 120.0, 121.5, 137.1, 149.7, 175.2.

PVP-glutathione dithio-conjugate 24

L-glutathione **23** (40 mg, 0.12 mmol) was added to a solution of PVP derivative **8** (80 mg, 0.02 mmol) dissolved in deoxygenated TRIZMA® HCl buffer solution pH = 8 (2 mL), and the reaction

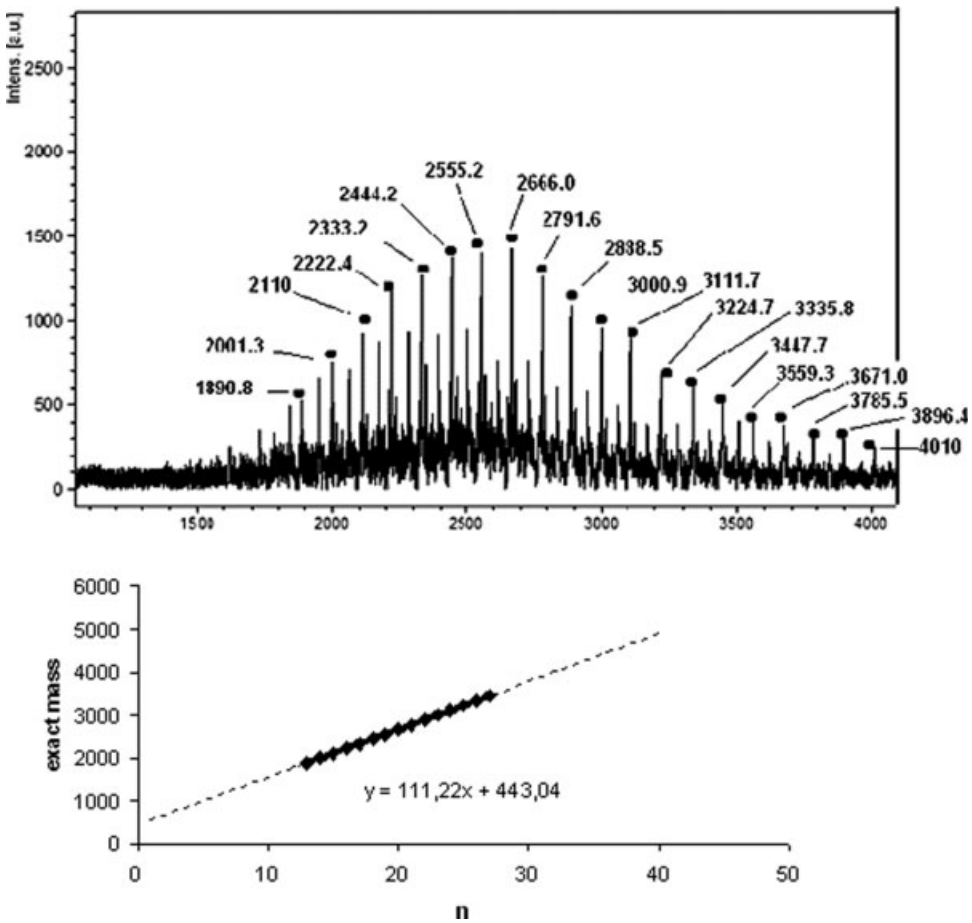


Chart 12. Calc.: y = 111.07x + 452.14; Exp.: y = 111.22x + 443.04 (error ~ 2%).

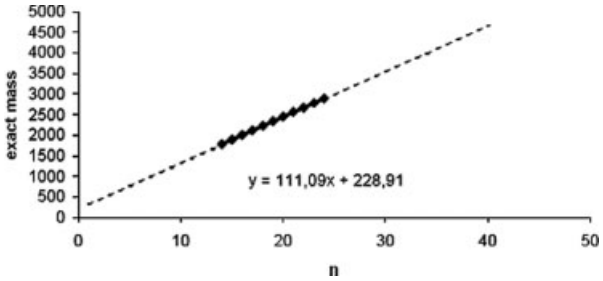


Chart 13. Calc.: 111.07x + 210.10; Exp.: 110.92x + 228.91 (error ~ 2%).

mixture was stirred for 3 days at room temperature. The solution was diluted with water (30 mL) and directly ultrafiltered. Polymer **24** was recovered as a white powder.

^{1}H NMR ($CDCl_3$) δ (ppm) = 1.39–1.69 (m, CH_2 + CH_3); 2.0 (m, CH_2 pyr); 2.34 (m, CH_2CO+ CH_2S); 3.20 (m, CH_2N); 3.69–3.80 (m, CH). ^{13}C NMR ($CDCl_3$) δ (ppm) = 18.3, 31.4, 34.6, 42.1, 43.6, 44.8, 175.4.

MALDI-TOF spectrum and end-group analysis of **24** (Chart 12).

PVP-aminoethylmaleimido carboxamide 11

HBTU (110 mg, 0.28 mmol) and DIEA (0.1 mL, 0.58 mmol) were added to solution of PVP-COOH **1** (260 mg, 0.058 mmol) in dry DMF (2 mL). The mixture was stirred for 5 min at room temperature then a solution of **25** (80 mg, 0.29 mmol)[51] in DMF (1 mL) was added. The reaction was stirred for 3 days at the same temperature. Product **11** was isolated as a slight yellow powder.

^{1}H NMR ($CDCl_3$) δ (ppm) = 1.44–1.71 (m, CH_2 + CH_3); 2.04 (m, CH_2 pyr); 2.25–2.37 (m, CH_2CO); 3.25 (m, CH_2N); 3.73–3.89 (m, CH); 6.66–6.68 (m, CH maleimide). ^{13}C NMR ($CDCl_3$) δ (ppm) = 18.2, 31.3, 34.6, 35.4, 42.0, 43.6, 44.8, 134.0, 175.0.

MALDI-TOF end-group analysis of **11** (Chart 13).

PVP-glutathione maleimido conjugate 26

L-glutathione **23** (70 mg, 0.2 mmol) was added to a solution of PVP derivative **11** (90 mg, 0.02 mmol) dissolved in deoxygenated TRIZMA® HCl buffer solution pH = 8 (2 mL), and the reaction mixture was stirred for 3 days at room temperature. The yellowish solution was diluted with water (30 mL) and directly ultrafiltered. Polymer **26** was recovered as a white powder.

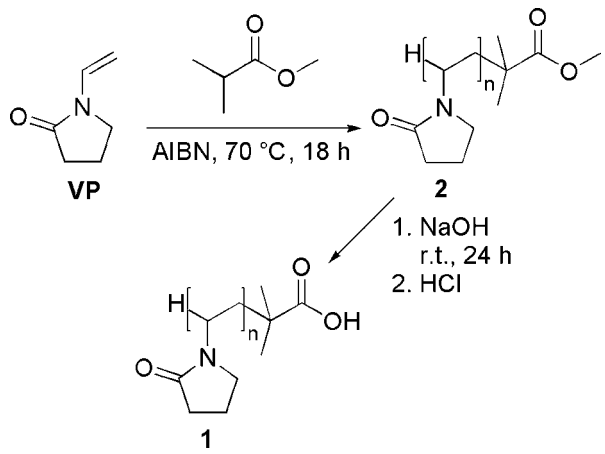
^{1}H NMR ($CDCl_3$) δ (ppm) = 1.44–1.71 (m, CH_2 + CH_3); 2.04 (m, CH_2 pyr); 2.25–2.37 (m, CH_2CO); 3.25 (m, CH_2N); 3.73–3.89 (m, CH). ^{13}C NMR ($CDCl_3$) δ (ppm) = 18.2, 31.3, 34.6, 35.4, 42.0, 43.6, 44.8, 175.0.

RESULTS AND DISCUSSION

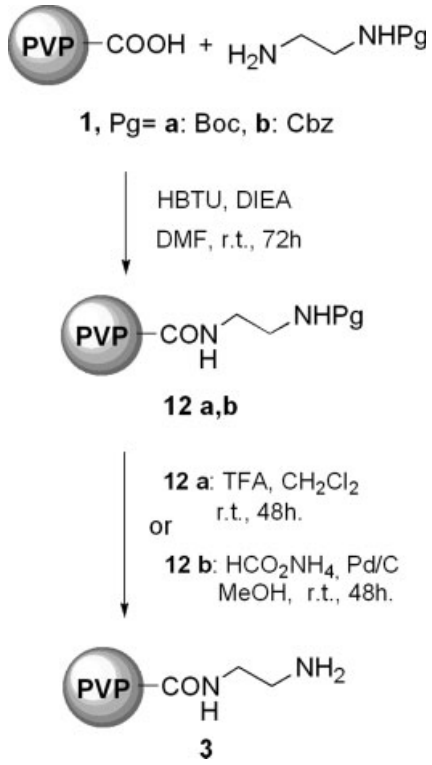
The starting polymer PVP-COOH **1** ($\overline{M}_n$ = 4535, see "Experimental" section) was synthesized, as previously reported by Ranucci et al, by chain-transfer radical polymerization of 1-vinylpyrrolidone (VP) in the presence of methyl isobutyrate, as chain transfer agent (CTA) (Scheme 2). PVP-carbomethoxy derivative **2** thus obtained was subsequently hydrolyzed with NaOH to give **1**.[31,32]

PVP-COOH **1** was then reacted, in the presence of a condensing agent, with the amino group of different bifunctional reagents, to produce a series of new PVP derivatives **3–11** (Scheme 1) bearing different terminal groups. All these reactions went easily to completion and the new derivatives were characterized by MALDI-TOF analysis (see "Experimental" section).[40,41]

The first reaction was carried out on mono-protected (Boc or Cbz) ethylenediamine, in DMF using diisopropylethyl amine (DIEA) and *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) as condensing agent, stirring the mixture at room temperature for 3 days (Scheme 3). The corresponding *N*-protected 2-aminoethyl carboxyamide poly-



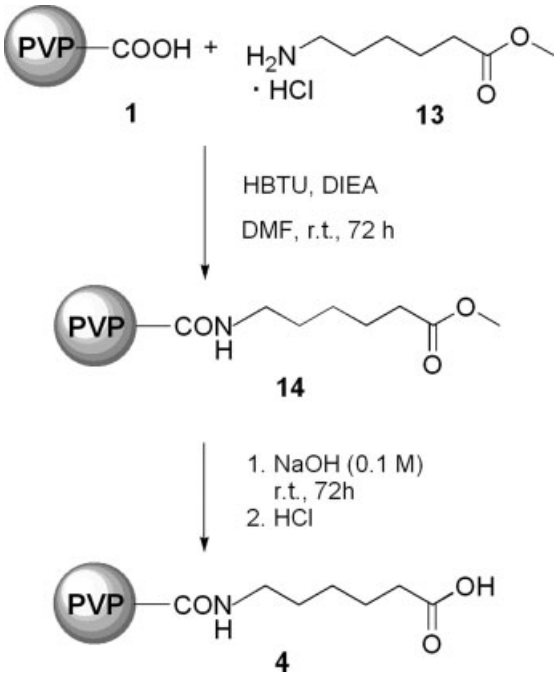
Scheme 2. Synthesis of PVP-COOH **1**.



Scheme 3. Synthesis of NH_2-terminated PVP **3**.

mers **12 a** or **b** were isolated. The Boc or Cbz group was then removed with trifluoroacetic acid (TFA) in CH_2Cl_2 at room temperature in the case of **12 a** or by hydrogenolysis for **12 b** to give NH_2-terminated PVP **3**.

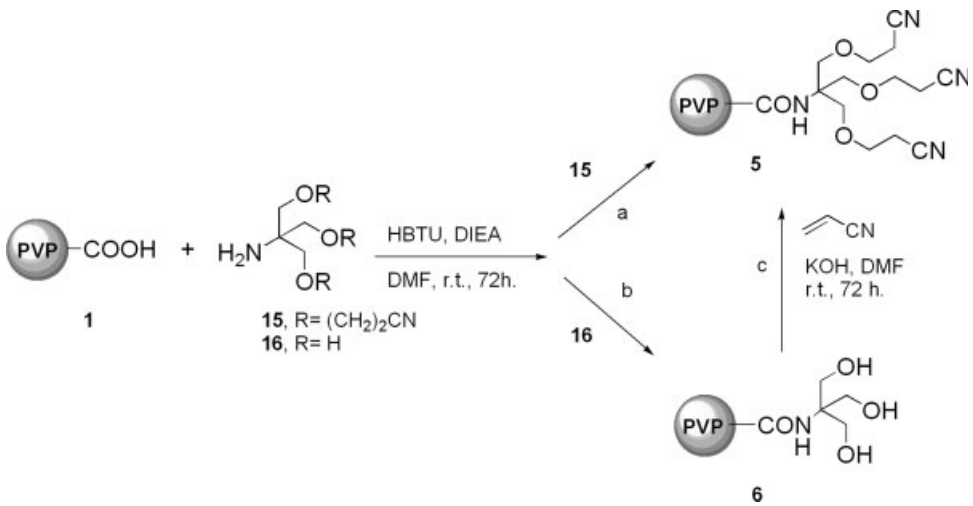
In the same reaction conditions, PVP-COOH **1** was reacted with 6-amino methyl hexanoate hydrochloride **13** (Scheme 4).



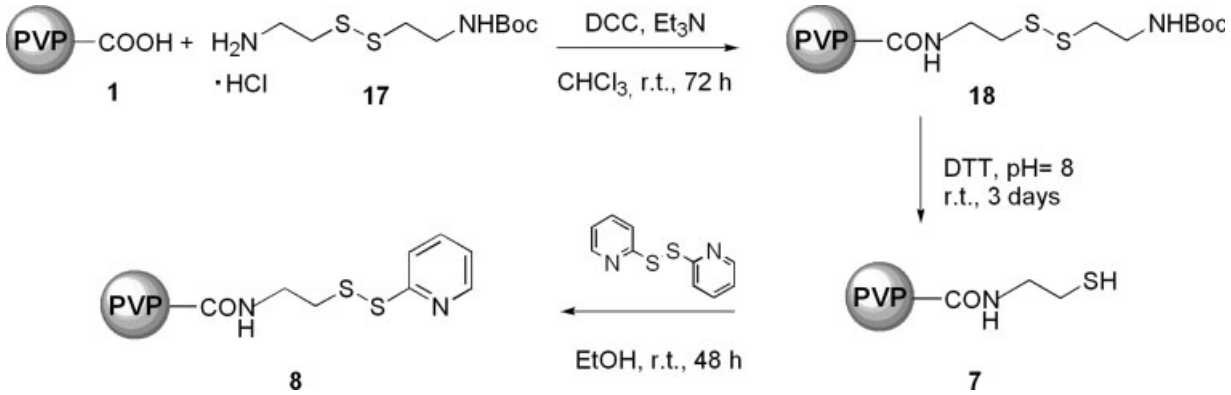
Scheme 4. Synthesis of hexanoic acid PVP derivative **4**.

The corresponding PVP methyl ester **14** was isolated and then hydrolyzed with 0.1 M NaOH solution, subsequent acidification with HCl (0.1 M) produced carboxylic acid polymer **4**. In this polymer the long alkyl chain should keep the carboxyl group away from the core of the polymer thus reducing the steric hindrance, and this might be useful in the case of reactions with other bulky molecules.

With the aim of obtaining higher loading polymers, PVP-COOH **1** was also reacted with



Scheme 5. Synthesis of higher loading PVP derivatives **5** and **6**.



Scheme 6. Synthesis of pyridyl disulfide PVP derivative **8**.

poly-functionalized molecules, such as tris[(cyanoethoxy)methyl]aminomethane (**15**) (Scheme 5, path a) and tris(hydroxymethyl)aminomethane (**16**) (Scheme 5, path b). In both cases the condensations were again made in DMF at room temperature in the presence of HBTU and DIEA, the corresponding polyfunctionalized polymers **5** and **6** were isolated.

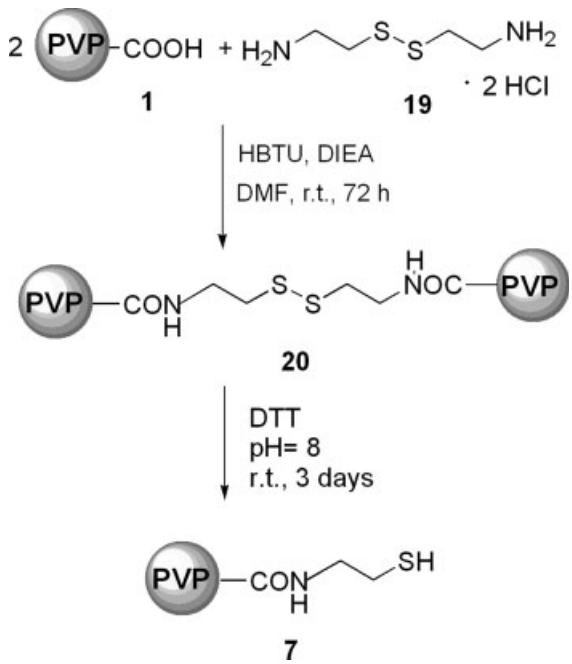
PVP **5** was also obtained from the Michael addition of polymer **6** to acrylonitrile (Scheme 5, path c) applying the same experimental conditions reported for the solution synthesis of **15**, namely the use of KOH in dioxane as solvent at room temperature. This latter way afforded polymer **5** in higher purity, in addition this reaction constitutes a promising test of the reactivity of such poly-functionalized PVP. Moreover the cyano groups in derivative **5** can be further transformed into amino or carboxylic functions thus extending the number of higher loading PVP derivatives.

The design of effective polymer drug conjugate needs in some cases the use of a linker, stable during the transport but able to release the drug at the target site, thus improving the drug action.[7,52] The polymers **8**, **10**, and **11** (Scheme 1) bearing an activated disulphide or a maleimido linker were specifically designed for this purpose allowing the linkage of SH-terminated molecules through the formation of a sulphide or disulphide bond. In a similar way thiol-terminated polymer **7** and **9** can be used in the reactions with properly functionalized biomolecules.

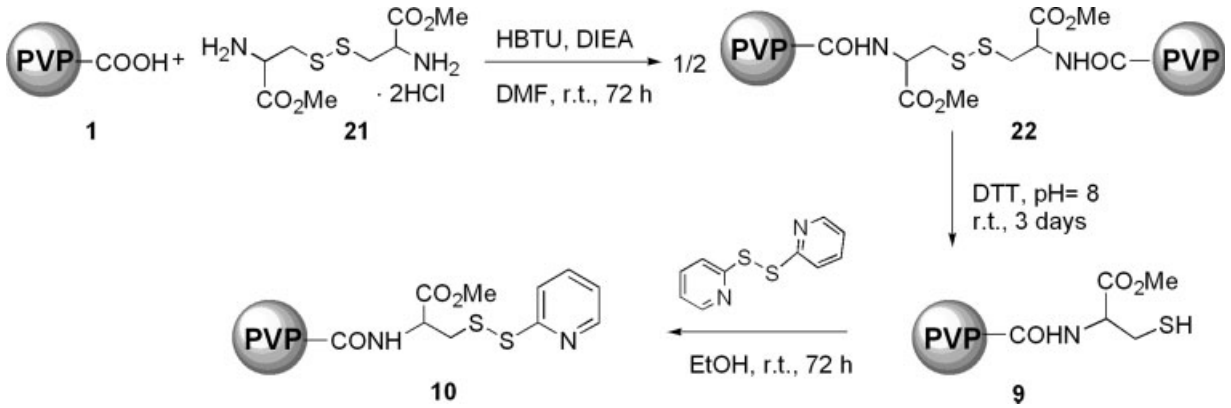
We first investigated the formation of the disulfide linkage, which results particularly useful because it allows the release of drugs in the reducing conditions of the endosomal compartment of a cell or by the action of SH-containing proteins.[52] Usually this bond is formed through the nucleophilic substitution of a thiol group on a S—S bond, activated by the presence of a good living group, that is, 2-mercapto pyridine. To this end PVP-COOH **1** was condensed with monoprotected *N*-Boc cysteamine **17** in the presence of dicyclohexylcarbodiimide (DCC) at room temperature in $CHCl_3$ to give disulfide derivative **18** (Scheme 6).

The reduction of disulfide by means of 1,4-dithiothreitol (DTT) in a buffer solution (TRIZMA® HCl, pH = 8) produced the SH-terminated polymer **7**, which was directly reacted with 2,2′-dithiopyridine to give pyridyl disulfide polymer **8**.

More conveniently PVP-SH **7** was easily obtained, in an "atom economy strategy," from the



Scheme 7. Synthesis of SH-terminated PVP **7**.



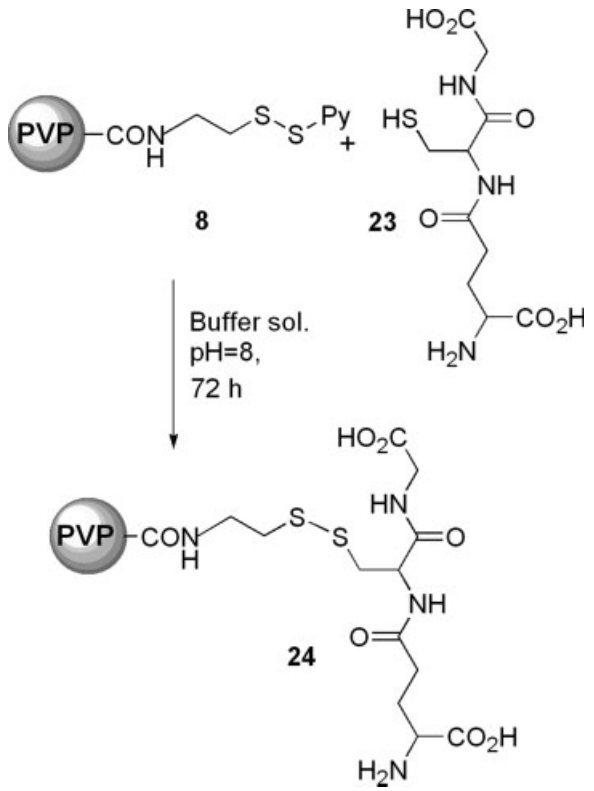
Scheme 8. Synthesis of cysteine-terminated PVP **9**.

reaction between **1** (2 equiv.) and unprotected cystamine **19** (Scheme 7), PVP disulfide **20**, was isolated and subsequently reduced with DTT to give the same thiol **7**.

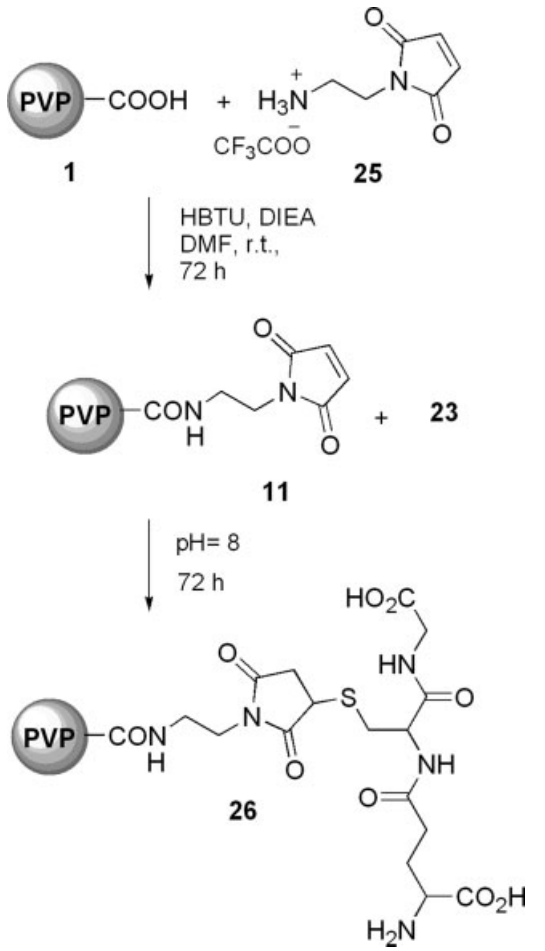
A similar synthetic strategy was also applied for obtaining cysteine terminated PVP (Scheme 8), by reacting PVP-COOH **1** (2 equiv.) with cystine hydrochloride **21** in the previous reaction conditions.

PVP disulfide **22** thus obtained was reduced to cysteine-terminated PVP **9**, which was then converted into the corresponding activated pyridine disulfide **10**.

PVP disulfide **22** thus obtained was reduced to cysteine-terminated PVP **9**, which was then converted into the corresponding activated pyridine disulfide **10**.



Scheme 9. Synthesis of glutathione-PVP "S-S conjugate" **24**.



Scheme 10. Synthesis of glutathione-PVP "maleimido conjugate" **26**.

Figure 1. PVP-COOH **1** extended conformation.

As previously mentioned, PVP disulfides such as **8** and **10** are suitable supports for the attachment of SH-containing molecules (i.e., peptides, proteins), thus we tested the reactivity of **8** toward a biologically active compound, namely glutathione **23**, which is short cysteine-containing peptide. The reaction was successfully run in aqueous buffer solution (pH = 8) for 3 days at room temperature and afforded glutathione-PVP conjugate **24** (Scheme 9).

A further possibility for the conjugation of SH-containing molecules to PVP is the use of a maleimido-functionalized polymer such as **11**, that was obtained through the reaction of PVP **1** with *N*-(2-aminoethyl)maleimide **25** (Scheme 10).[51]

Michael addition reaction of SH-group of glutathione **23** on maleimido double bond of polymer **11**, allowed to obtain the corresponding polymer-glutathione conjugate **26**.

At this stage of the work, we got a rather general picture of the reactivity of PVP-COOH **1** with small molecules and of the possibility of obtaining different end-terminated derivatives. The good reactivity of the carboxy group on the bulky PVP chain, suggests a favorable conformation of the polymer in solution, making the COOH available to the reagents. As mentioned earlier, just one article concerning the molecular modeling of PVP has been published,[39] nothing about conformational studies of functionalized PVP is known, and for this reason we decided to perform a computational study of PVP-COOH **1**, based on a molecular dynamic simulation. As neither crystallographic nor unique NMR structural information were actually available to choose a suitable starting conformation, we tackled the study herein reported as an *ab initio* folding problem.

It should be noted that, if compared with the folding of a peptide having a MW similar to our system, PVP should provide quite meaningful results in a shorter time, as conformational trapping in local minima should be reduced by the impossibility of the polymer to be stabilized by strong intramolecular hydrogen bonds. The molecular dynamic run was then conduced starting from an atactic PVP consisting of 40 monomers, including the modified acidic residue of PVP-COOH **1**. The completely extended starting conformation (Fig. 1) was then gradually heated for 50 ps to a final temperature of 325 K, and a production run was then conduced for 75 ns at a simulation temperature of 325 K. This temperature was chosen to minimize the probability for the system to be kinetically trapped in local minima, as this strategy was successfully adopted for a small protein folding study,[53] where a working temperature of 325 K resulted enough to provide a thorough sampling of the peptide conformational space, but avoiding system instabilities. To confirm the suitability of the chosen approach to our system, we performed two independent simu-

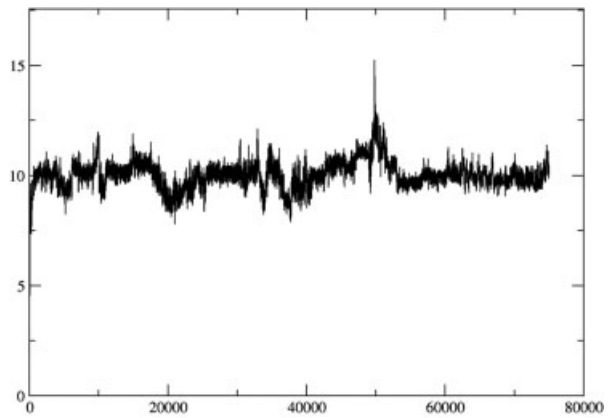


Figure 2. RMSD versus time (ps).

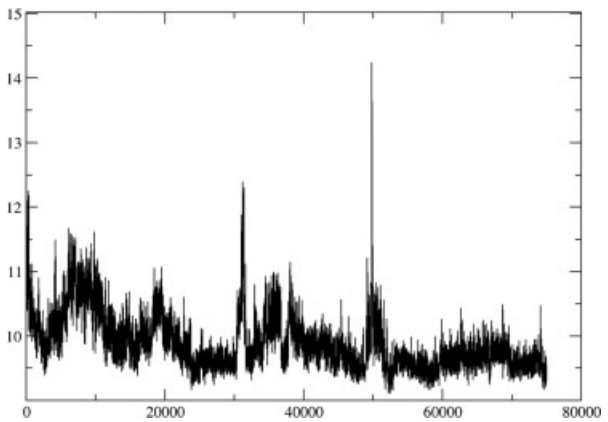


Figure 3. Radius of gyration versus time (ps).

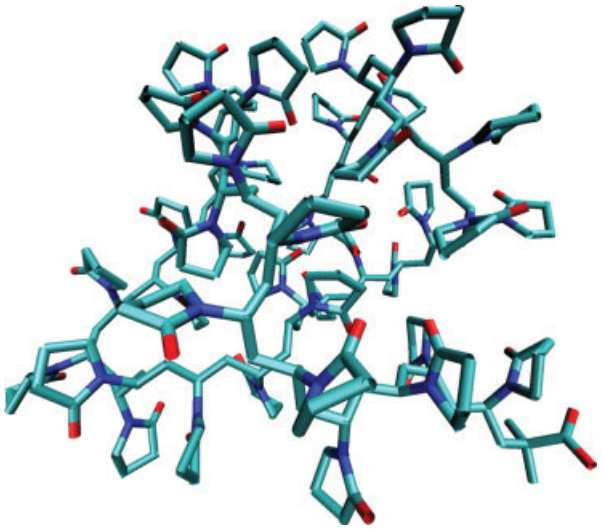


Figure 4. Minimum potential energy structure on the 75-ns PES.

lations and the trajectory analysis provided essentially identical results.

Thus, the molecular dynamic (MD) run was conduced for a total simulation time of 75 ns, in other words until two consecutive 10 ns runs did note provide consistent conformational changes, monitored in terms of root mean square deviation (RMSD). Indeed, as shown in Figures 2 and 3, both the plotted RMSD, showing the root mean square displacement in the structure atomic coordinates between time t and t_0, and the radius of gyration suggest that between 55 and 75 ns the system adopted a quite stable conformation, even if the lowest potential energy conformation (Fig. 4) was obtained at a simulation time of 26.25 ns.

However, the two peaks of the radius of gyration observed at about 30 and 50 ns show that

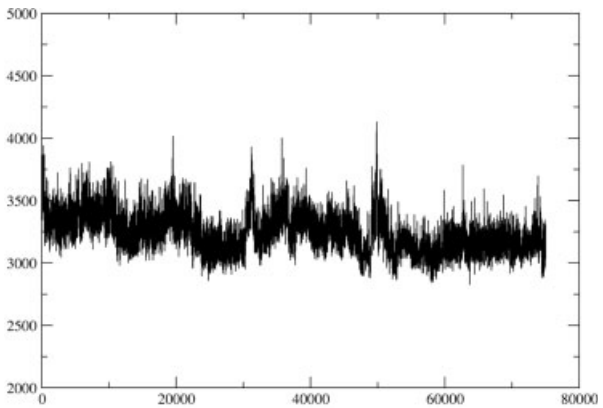


Figure 5. Solvent accessible surface area versus time (ps).

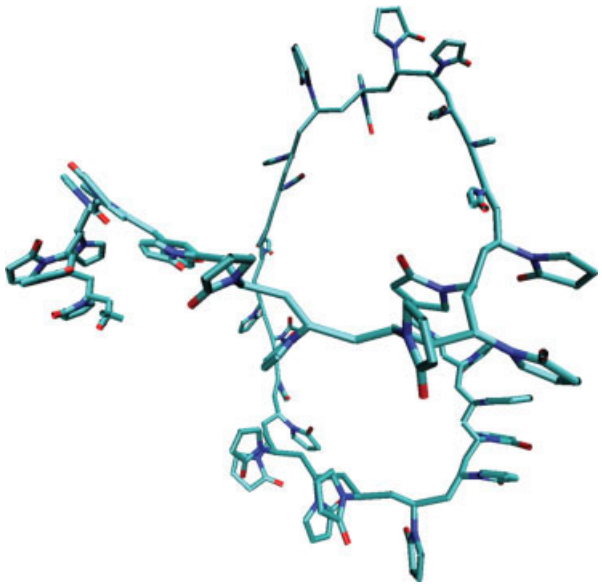


Figure 6. Averaged geometry along the whole trajectory.

the polymer structure went through consistent conformational changes, indicating a sort of a periodic folding–unfolding behavior, as can be expected by the lack of stabilizing intramolecular hydrogen bonds. This is also confirmed by the analysis of the variation of the solvent accessible surface area (SASA) during the MD run (Fig. 5). Moreover, the mean geometry obtained by averaging the coordinates along the whole trajectory (Fig. 6) shows a moderately unfolded structure. It should be noted that the carboxylic function results well exposed to the solvent in both the minimum energy and the averaged structures, thus explaining the good reactivity shown by experiments.

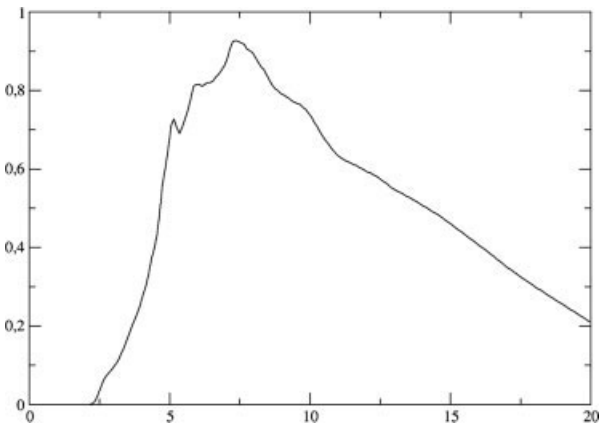


Figure 7. Radial distribution function on COOH.

To provide a further proof to the above observation we calculated the radial distribution function around the acidic moiety. This function, calculated on all the 75-ns simulation time, provides the probability to find any other residue within a certain distance from the center of mass of the carboxylic group. As shown in Figure 7, such a probability is null between 0 and 2.4 Å, it shows a first peak at about 5 Å and is maximum at about 7.5 Å, thus confirming the good exposure of the acidic function.

CONCLUSIONS

The main aim of this article was to widen the potential of end-carboxylated PVP oligomers as soluble carriers. We achieved this goal by performing a series of organic reactions leading to new functionalized PVP derivatives. All these reactions went easily to completion and the new derivatives proved amenable to coupling reactions with a number of model compounds, including for instance reduced glutathione.

The presence of the bulky PVP chain did not hamper the reactivity of the carboxyl group. This would imply that in solution the carboxyl group is not buried inside the coil, but well exposed to the solvent. A molecular dynamics conformational study confirmed this, further demonstrating the considerable potential of end-carboxylated PVP as the starting point for preparing soluble conjugates of biologically active molecules.

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