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The thermodynamics of basic and amphoteric poly(amido-amine)s containing peptide nitrogens as potential binding sites for metal ions. Part 1

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Abstract

Five basic and amphoteric poly(amido-amine)s containing, besides carboxyl and tertiary amino groups, peptide nitrogens were synthesised by a Michael-type polyaddition reaction of a primary (*N*-methylamine) or a bis-secondary (*N*,*N*′-dimethyl-ethylenediamine) amine to flexible bis-acrylamides.

Protonation and copper(II)-complex formation studies were performed in 0.1 M NaCl at 25°C by potentiometry, calorimetry, viscometry and UV–Visible spectroscopy. Compared with the previously studied homologous PAAs, having the rigid bis-diacylpiperazine ring inserted in the monomer unit, the basic polymers showed lower basicity constants and increased polyelectrolyte behaviour. In fact, the *n* parameter of the modified Henderson–Hasselbalch equation was always higher than 1, suggesting reduced shielding among repeating units of the polymer. The presence of charges (positively ionised nitrogens or negative carboxylate groups) produced almost unitary *n* values because of enhanced shielding due to the larger hydration shell surrounding the ionised groups. In contrast, neutralization of ampholytes to zwitterions raised the *n* values owing to the release of water molecules, which increased the entropy contribution ($\Delta S°$) and compacted the macromolecule.

Copper(II) ions were easily complexed by amino nitrogens in a stable five-member ring showing three complex species of different stoichiometry [CuL, CuH(−1)L and CuH(−2)L] (L means the repeating unit of the polymer) on the whole range of pH investigated (3–11). Thermodynamic ($\log \beta$, $-\Delta H°$, $\Delta S°$) and UV–Visible data are in line with the proposed complex species that also involve deprotonation of one or two peptide nitrogens in different pH-ranges. Polyampholytes were able to bind copper(II) ions through peptide sites at low pHs (>3), while the basic PAAs needed to reach pH > 5. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Poly(amido-amine)s; Protonation; Metal complexes

1. Introduction

The protonation behaviour of polyelectrolytes and their complex formation with heavy metal ions have been widely studied in the past [1–3]. Poly(amido-amine)s (PAAs), which are characterised by the regular arrangement of amido and tertiary amino groups along their chain, are among the polymers mainly studied in this respect [3–5]. PAAs are an interesting class of water-soluble polymers that, owing to complexation capability towards metal ions, are being considered for removal of heavy metal ions from waste-waters [6,7]. The low cytotoxicity [8,9], the degradability [10] as well as the possibility of binding drugs to these polymers have encouraged their use in the biomedical field also [2]. For instance some PAAs have been shown to bind heparin reversibly at physiological pH [11] while others have exhibited a potential as vectors for intracytoplasmic delivery of nucleic acids [8,12] or as drug carrier polymers [9,13]. In the first two cases macromolecular complexes stabilised mainly by electrostatic interactions are formed. Therefore the degree of protonation affects complex stability dramatically.

With regard to intracytoplasmatic delivery, macromolecules as a rule enter the cell by endocytosis. As a consequence they are exposed to pH changes from pH 7.4 extracellularly to pH 5.5–6.5 intracellularly within the endosomal–lysosomal compartment. Interestingly, some PAAs exhibit pH-dependent conformational transitions over a narrow pH range [14]. Therefore, PAAs with basicity constants that ensure transitions to more expanded coils in correspondence to pH changes from 7.4 to 5.5–6.5 can be

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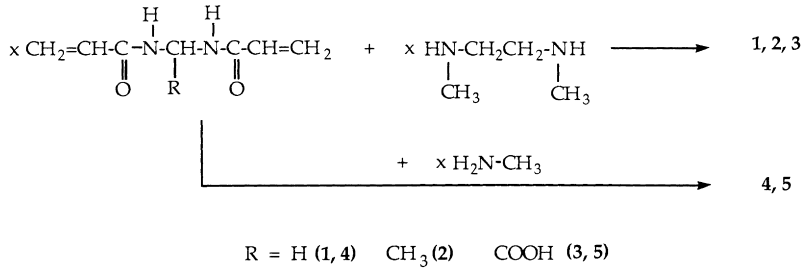
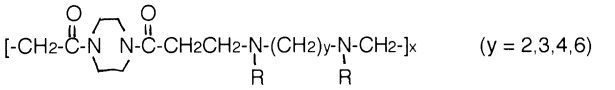


Scheme 1.

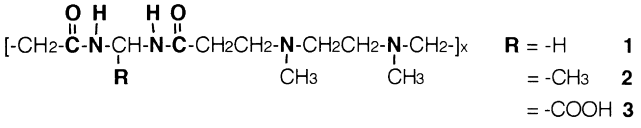
prepared by selecting their structures properly. Then, in principle, bound drugs are protected by the folded macromolecular chain during transport in extracellular fluids at pH 7.4 while they are exposed or liberated in the intracytoplasmatic milieu after cell internalization.

In spite of the variety of PAA structures that can be prepared, so far most physico-chemical investigations have been confined to PAAs containing the rigid diacylpiperazine moiety [4,15]



The latter acts as an effective shielding group of vicinal monomer units making the thermodynamic properties of these polymers *real*, i.e. the basicity and stability constants of each repeating unit are independent of each other [4,14,16]. In some cases the introduction of carboxylate groups through amino-acid residues results in a reduction of this peculiar behaviour, nevertheless a unitary stoichiometric ratio between the number of repeating units and metal ions is always observed [3,17,18].

In this paper we wish to report on the protonation behaviour and copper(II)-complex formation of a series of synthetic PAAs having higher macromolecular flexibility



that were synthesised by reaction of *N*,*N*′-dimethyl-ethylenediamine (DMEDA) with three different flexible bisacrylamides and were studied in aqueous media by thermodynamic (viscometry, potentiometry, solution calorimetry) and spectroscopic (UV–Visible) techniques.

Owing to the absence of ring structures along the chain, these polymers are expected to possess higher macromolecular flexibility compared to the corresponding diacylpiperazine PAAs from which they differ also for the presence of peptide linkages along the main chain. Both tertiary amino nitrogens will be involved in the coordination process with the metal ion, thus forming a stable five-membered ring as observed in homologous polymers considered so far [4,5,7].

However, it is expected that peptide nitrogens may act as potential binding sites for copper(II) ions, thus contributing

Table 1
Structure of poly(amido-amine)s

Polymer	Structure	
1	-CH2-C-N-CH2-N-C-CH2CH2-N-CH2CH2-N-CH2- (CH3, CH3)	MBA-DMEDA
2	-CH2-C-N-CH-N-C-CH2CH2-N-CH2CH2-N-CH2- (CH3, CH3, CH3)	EBA-DMEDA
3	-CH2-C-N-CH-N-C-CH2CH2-N-CH2CH2-N-CH2- (COO⁻, CH3, CH3)	BAC-DMEDA
4	-CH2-C-N-CH2-N-C-CH2CH2-N-CH2- (CH3)	MBA-MMA
5	-CH2-C-N-CH-N-C-CH2CH2-N-CH2- (COO⁻, CH3)	BAC-MMA

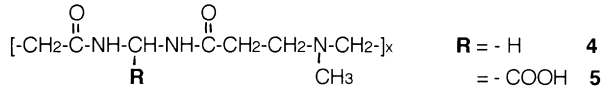
Table 2
Molecular weight characterization of polymers

Polymer	$[\eta]$[a]	$\bar{M}_w$[b]	$\bar{M}_n$[c]	d[d]
1	0.49	34 600	22 900	1.5
2	0.37	26 600	18 300	1.5
3	0.39	46 300	29 600	1.6
4	0.23	11 300	8100	1.4
5	0.21	14 800	11 100	1.3

[a] Intrinsic viscosity (dl/g) at 30°C in Tris buffer pH 8.09.
[b] Weight-average molecular weight, calculated from GPC.
[c] Number-average molecular weight, calculated from GPC.
[d] Dispersity index, calculated from GPC.

to elucidate the behaviour of copper-containing biological molecules [19,20]. The presence of either hydrophobic (methyl) or hydrophylic (carboxyl) groups inserted between the two amido groups should further clarify the deprotonation mechanism of the peptide nitrogen in virtue of their inductive effect.

Finally, structurally related PAAs obtained by polyaddition of *N*-methylamine (MMA) to the same bis-acrylamides



were also investigated to compare the complexing behaviour towards the metal ions when only one tertiary basic nitrogen is inserted in the monomer unit.

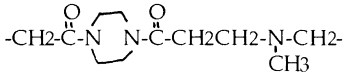
2. Results and discussion

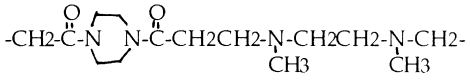
2.1. Synthesis

PAAs were synthesised by a Michael-type polyaddition reaction of either DMEDA or MMA to different bis-acrylamides, namely methylene-*N*,*N*′-bis(acrylamide) (MBA), 2,2′-bis(acrylamido)acetic acid (BAC) and *N*,*N*′-ethylidenebis(acrylamide) (EBA) (Scheme 1).

The polymerisation was carried out in water at 25°C under nitrogen atmosphere, with reaction time of 4 days. The structure of the five PAAs is reported in Table 1. Since the degradation rate of PAAs is known to increase with pH and temperature [10], aqueous hydrochloric acid solution was finally added to the solutions and the polymers were purified by ultrafiltration in cold water (10°C). A molecular weight characterisation of the samples was performed both by viscometry and gel permeation chromatography (GPC). From the GPC traces the average molecular weights and the dispersity index of the different PAAs were calculated using the calibration curve determined for the PAA deriving from the polyaddition of 2-methylpiperazine to 1,4-bis(acryloyl)piperazine (BAP) [21]. The results are summarised in Table 2. The trend of intrinsic viscosities

Table 3
Protonation: thermodynamic functions of poly(amido-amine)s at 25°C in 0.1 M NaCl

PAA1:


PAA2:


Polymer	Reaction step	log K°[a]	n[a]	$-\Delta G^{\circ}$ kJ/mol	$-\Delta H^{\circ}$ (kJ/mol)	ΔS°[b] (J/mol K)	Ref.
1	1	7.77(5)	1.13(3)	44.4(3)	26.2(3)	61(1)	This work
	2	4.28(5)	1.02(3)	24.4(3)	24.2(3)	1(1)	
2	1	7.83(6)	1.20(4)	44.7(3)	27.0(8)	59(3)	This work
	2	4.29(9)	1.04(4)	24.5(5)	25.0(8)	−2(2)	
3	1	8.52(5)	1.10(7)	48.6(3)	26.2(2)	75(1)	This work
	2	4.85(9)	1.20(4)	27.7(6)	24.4(7)	11(2)	
	3	2.0(3)	–	11(2)	–	–	
4	1	7.21(3)	1.16(2)	41.1(2)	30.3(1)	36.4(4)	This work
5	1	8.04(5)	1.24(3)	45.9(3)	28.2(6)	59(2)	This work
	2	2.0(4)	–	11(2)	–	–	
PAA1	1	7.79	–	44.4	34.6	33	[23]
PAA2	1	8.09	–	46.2	30.8	51	[23,24]
	2	4.54	–	25.9	25.5	1	

[a] $\log K = \log K^{\circ} + (n-1)\log[(1-\alpha)/\alpha]$; $(-\Delta G^{\circ} = RT \ln K)$; figures in parentheses are standard deviations.
[b] $\Delta S^{\circ} = (\Delta H^{\circ} - \Delta G^{\circ})/T$, calculated at $\alpha = 0.5$.

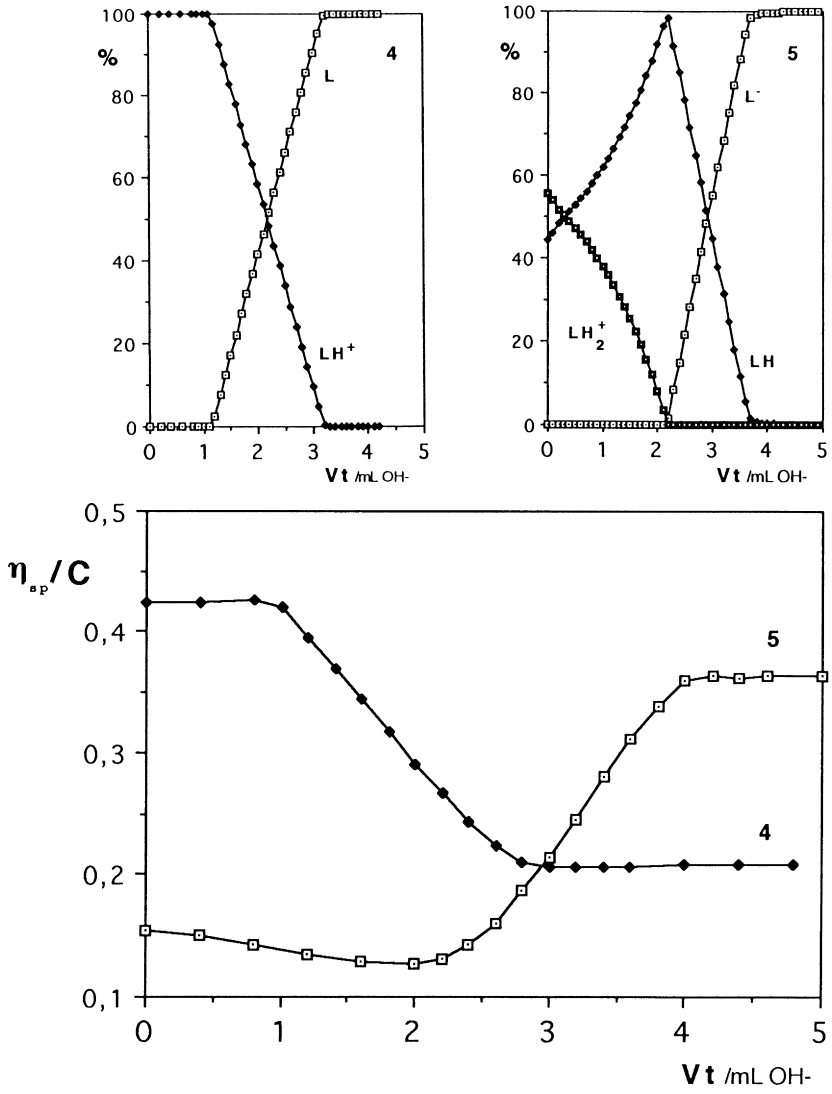


Fig. 1. Reduced viscosity (η_{sp}/C, dl/g) and species distribution curves (%) in relation to the volume (V_t, ml) of titrant (0.1 M NaOH) added to acidified solutions (25 ml 0.1 M NaCl) of polymers **4** (conc. 0.17 g/dl) and **5** (conc. 0.17 g/dl) at 25°C.

is in qualitative agreement with the GPC data, but polymers **3** and **5**, which are derived from BAC, have higher molecular weights than expected on the basis of their viscosity. This is a consequence of the amphoteric nature of these PAAs which, owing to attractive interactions between carboxylate and onium ions, is likely to produce a more compact coil in comparison with the related polymers derived from MBA and EBA. The molecular weights of **4** and **5** are lower than those of PAAs synthesised from DMEDA, reflecting at least in part the lower weight of their repeating units.

FT-IR spectra, obtained by casting from aqueous solutions, are in agreement with the polymer structures and are dependent on the pH of the medium; for instance, the wavenumber of the amide I band increases with decreasing pH because of the withdrawing effect exerted by protonated nitrogens [22]. If the spectra are recorded at pH 11, where all PAAs are completely deprotonated, the amide I band turns out to be located at 1651 cm^{-1} in **1** and **4**; in the polymer derived from BAC this band is displaced at a lower wavenumber (1634 cm^{-1}) while in the PAA prepared from EBA its wavenumber is increased (1661 cm^{-1}). This suggests that carboxylate and methyl groups exert an opposite inductive effect, with the latter acting as an electron-donating group. The proposed structure was also confirmed by proton NMR analysis. In all cases, the observed broad lines for the backbone and side-chain resonances are consistent with a slowly tumbling macromolecular species in solution.

2.2. Thermodynamics of protonation

The basicity constants (log K) and enthalpy changes ($-\Delta H°$) for the protonation of polymers were determined at 25°C in 0.1 M NaCl. Table 3 summarises the experimental results together with the calculated entropy changes

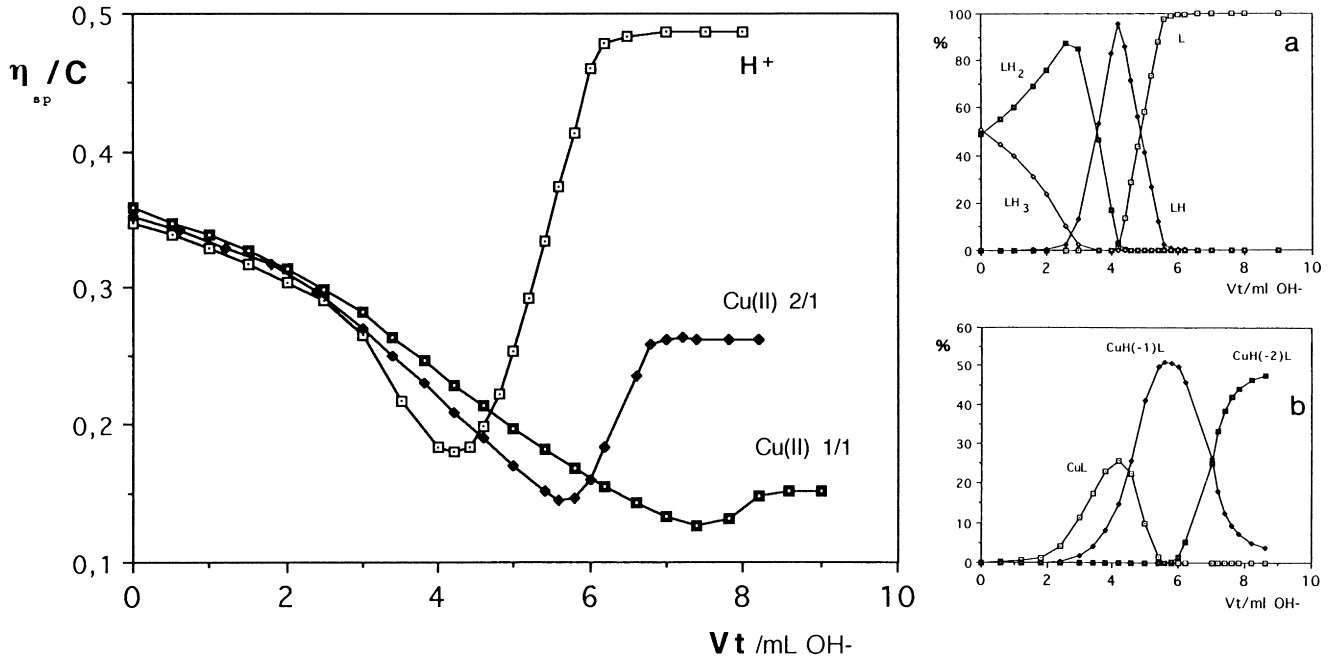


Fig. 2. Reduced viscosity (η_{sp}/C, dl/g) in relation to the volume (V_t, ml) of titrant (0.1 M NaOH) added to acidified solutions (25 ml 0.1 M NaCl) of the polymer **3** in the absence (conc. 0.24 g/dl) and in the presence (conc. 0.22 g/dl) of copper(II) ions [Cu(II)/L molar ratios of 2 and 1] at 25°C. Inserted species distribution curves (%) for: (a) protonation; and (b) Cu(II)–complex formation of polymer **3** with the L/Cu(II) molar ratio of 2, as calculated by the stability constants.

($\Delta S°$). In the same table the corresponding functions of two related "traditional" PAAs (PAA1 and PAA2) are reported for comparison [14,23,24].

The polymers under study exhibit to some extent a polyelectrolyte behaviour, their basicity constants being on the borderline between the *real* and *apparent* values. In fact, the *n* parameter of the modified Henderson–Hasselbalch equation [5]

$$\log K = \log K° + (n - 1)\log[(1 - \alpha)/\alpha]$$

is generally higher than 1, meaning that the protonation of the basic sites becomes more difficult as the degree of protonation α increased. Alkalimetric titrations always showed curves with a sharp break-point and two well-defined buffer regions for polymers **1**, **2** and **3**. In contrast, polymers **4** and **5** revealed only one buffered region. The break-point allowed the precise determination of the amount of ionisable sites useful for the evaluation of reliable basicity constants. Unlike traditional PAAs, showing *real* thermodynamic functions for the presence of a rigid and effective shielding group like the acyl-piperazine moiety, the results obtained for these PAAs are indicative of a lower shielding exerted on the monomer units by the shorter and more flexible amide moiety [16,25]. The relative magnitude of the *n* values in successive steps of protonation is very noteworthy. For polymers **1** and **2**, $n_1 > n_2$ because the first step of protonation produces an onium ion that contributes to enhance the shield between neighbouring units. In fact n_2 being close to 1 renders log K_2 practically independent of α, i.e. a *real* termodynamic function, as observed in traditional PAAs [4,5]. This can be ascribed to the large hydration shell surrounding the positively charged groups that improves shielding [16], as also confirmed by the behaviour of polymer **3**. The latter, in fact, shows a reversed pattern, $n_2 > n_1$. Negative charges, present on the skeleton of the polymer, contribute to shield neighbouring charges. Once protonated, the zwitterions lose net charges and the polyelectrolyte behaviour increases with n_2. Nothing can be said about the third protonation step in polymer **3** because of unreliable data. In fact, carboxylate groups are protonated only to a limited extent in the investigated range of pH due to their very low basicity [26]. In general, the greater chain flexibility results in lower log *K*s. Polymers **1** and **2**, which show striking similarity, have log *K*s sensibly lower than the structurally related traditional PAA2 [7,23]. This is the usual pattern observed when comparing polymers belonging to these series [25]. However, the presence of negative charges as in polymer **3** provokes greater log *K*s. This is attributed to the increased electric potential around the basic sites undergoing protonation. Following the first step of protonation, the internal charge neutralisation of the zwitterion enables even definitely higher log *K* values than in PAA2 because of the absence of a net positive charge.

Electrostatic interactions plays a key role in defining the conformational characteristics of the basic and amphoteric polymers. Fig. 1 shows comparative viscometric titration data of the simple polymers **4** and **5**. The reduced viscosity (η_{sp}/C) of the acidified polymer solution in relation to the titrant volume (sodium hydroxide solution) suggests a different conformational behaviour for them. Unlike

Table 4
Copper(II)-complex formation: thermodynamic data of poly(amido-amine)s at 25°C in 0.1 M NaCl (L is the monomer unit of the polymer, $-\Delta G^\circ = RT \ln \beta$, $\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T$; figures in parentheses are standard deviations)

Polymer	Complex species	log β	$-\Delta G^\circ$ (kJ/mol)	$-\Delta H^\circ$ (kJ/mol)	ΔS° (J/mol K)	Ref.
1	CuL^{2+}	7.53(2)	43.0(1)			This work
	$CuH(-1)L^{+}$	1.48(2)	8.4(1)			
	$CuH(-2)L$	−8.37(3)	−47.8(2)			
2	CuL^{2+}	8.07(5)	46.0(3)	26(1)	67(4)	This work
	$CuH(-1)L^{+}$	2.18(4)	12.4(2)	−28.3(8)	79(3)	
	$CuH(-2)L$	−7.41(5)	−42.3(3)	–	–	
3	CuL^{+}	9.37(4)	53.5(2)	26.4(8)	91(3)	This work
	$CuH(-1)L$	5.67(2)	32.4(1)	9.6(4)	76(1)	
	$CuH(-2)L^{-}$	−4.66(4)	−26.6(2)	–	–	
5	$CuH(-1)L$	1.27(1)	7.24(4)	−9.1(9)	13.1(7)	This work
	$CuH(-2)L^{-}$	−8.46(2)	−48.3(1)	−44(1)	−13(3)	
PAA2	CuL^{2+}	8.96	51.1	33.5	59	[23,24]
	$Cu(OH)L^{+}$	5.52	31.5	11.7	66	

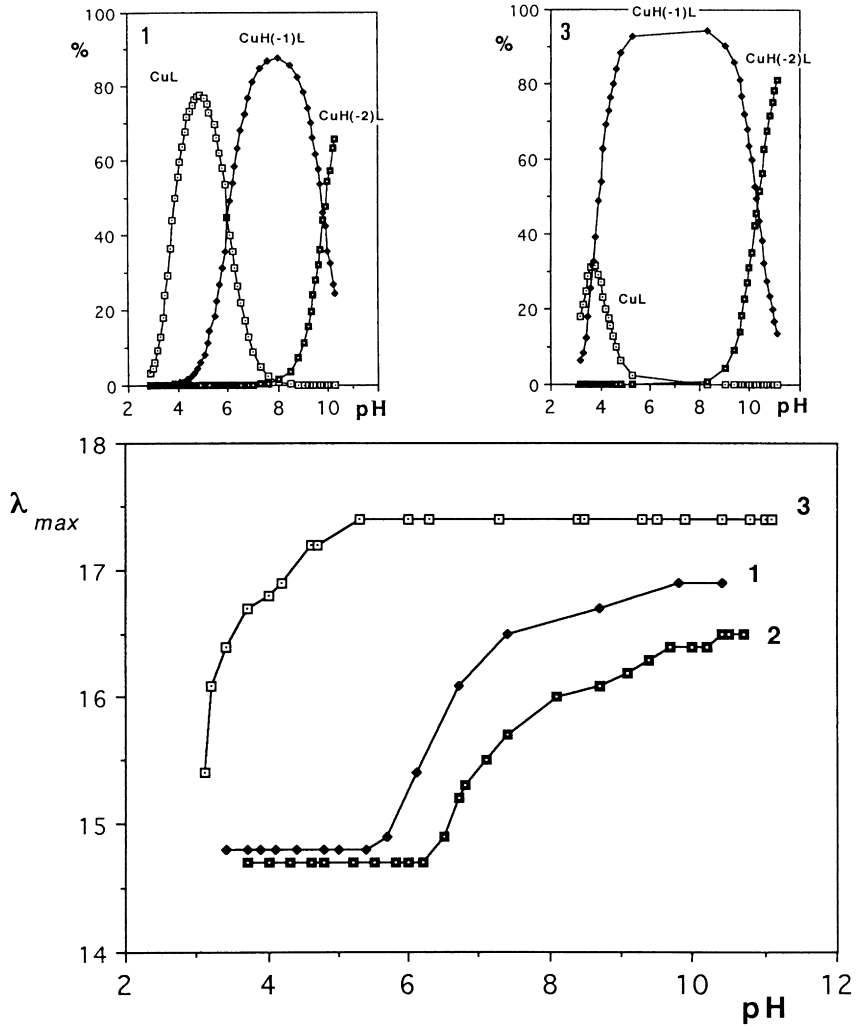


Fig. 3. Maximum wavelenghts (λ_{max}, kK/$\lambda_{max} \times 10^4$) in relation to pH for Cu(II)–complex species of polymers **1**, **2** and **3** in 0.1 M NaCl at 25°C. Inserted species distribution curves (%) for Cu(II)–polymer **1** and **3**, as calculated by the corresponding stability constants.

polymer **4**, showing a regular decrease of its coil dimension upon charge neutralization, the amphoteric polymer **5** reveals a minimum value of η_{sp}/C in correspondence to the maximum formation of zwitterionic species. Beyond such a value viscosity increases further due to lateral carboxylate anions that increase electrostatic repulsions more effectively than the charged amino groups on the main chain [27]. A similar behaviour was found for polymers **1**, **2** and **3** even if the presence of two protonated amino nitrogens per repeating units enhances the electrostatic effects. In particular, for the amphoteric polymer **3** a much more pronounced minimum in viscosity is observed. This occurs when the zwitterionic LH^{+-} species reaches its maximum value. A similar trend is observed also in the presence of metal ions (Fig. 2).

Calorimetric data reveal comparable enthalpy changes for both step of protonation in all the investigated PAAs. In all cases $-\Delta H^{\circ}$ values are *real*, even though the dependence of log K on α is considered [16]. This means that the variation of the basicity constants with pH is entirely due to entropy effects, simply related to the decrease in the freedom of retaining different conformations upon protonation [5,16]. The protonation of the first basic nitrogen is always more exothermic. However, the exothermicity is lower of about 4 kJ/mol compared to PAA2 [23,24] (Table 3). This difference may be ascribed to the H-bonding interaction between the onium ions and neighbouring amido C=O groups present in the latter case [14]. For the PAAs investigated, we can probably exclude the H-bonding interactions for the higher macromolecular flexibility of repeating units. The effect of protonation is reflected by the entropy contribution. Unlike the similar behaviour of polymer **1** and **2**, polymer **3** shows greater ΔS° values, reasonably associated with the release of water molecules around the basic sites undergoing protonation. This higher ΔS° value is attributed to the larger shell of water molecules surrounding the carboxylate groups that are lost during the first step of protonation to give zwitterions of low water content [26,27].

2.3. Copper(II) complex formation

The complexing ability towards the copper(II) ions was studied only for polymers **1**, **2**, **3** and **5**. Polymer **4** did not form complex species over the whole range of pH investigated (from 3 to 11) and the addition of metal ions in the alkaline region always led to precipitation of copper hydroxide prior to the formation of complex species, even the ligand/metal molar ratios were high. Table 4 summarises thermodynamic data obtained at 25°C in 0.1 M NaCl, whereas Fig. 3 shows a plot of the maximum wavelength λ_{max} in relation to pH for the complex species formed with polymers **1**, **2** and **3**. Unlike the flat trend of **5**, visible spectra of **1**, **2** and **3** clearly show a λ_{max} blue-shift increasing with pH. For PAAs derived from DMEDA, λ_{max} varies from 14.8 kK up to 17.4 kK whereas for **5**, λ_{max} has practically a constant value of 16.0 kK. This behaviour clearly suggests the involvement of further coordinating nitrogen atoms arising from deprotonation of one or two peptide groups. The polymers form three complex species of CuL, CuH(−1)L and CuH(−2)L stoichiometry [L is the monomer unit, H(−1)L and H(−2)L means the half and complete deprotonated forms, respectively, of the peptide moiety] that fit well the potentiometric titration curves by SUPERQUAD, a well-known program for small molecular-weight compounds [28]. In all cases, the stability constant log β for the two species involving peptide hydrogen deprotonation also are relative to the following reactions:

$$Cu^{2+} + L \rightleftharpoons CuH_{-1}L + H^{+}$$

and

$$Cu^{2+} + L \rightleftharpoons CuH_{-2}L + 2H^{+}$$

Polymer **5** forms only two complex species, CuH(−1)L and CuH(−2)L, between pH 4 and 11. Even if this polymer has only one tertiary amino nitrogen per repeating unit, it is able to form complex species by virtue of the easy deprotonation of the neighbouring peptide nitrogens [29]. The deprotonation process is enhanced by the presence of the carboxyl group, having electron withdrawing properties. This does not occur in polymer **4**, which does not form stable complexes. It is worth mentioning that some structurally related amido-thioether polymers also, containing a carboxyl group inserted between the two adjacent peptide nitrogens form complex species of different stoichiometry [30]. It is evident that the basic nitrogens are essential for anchoring the metal ions and that further coordination positions are satisfied by neighbouring donor groups. These groups may include the carbonyl oxygen that is replaced by the peptide nitrogen as pH becomes greater. A comparison of the complexing behaviour of polymers **1**, **2** and **3** supports this experimental evidence. Polymer **3**, in fact, having two amino nitrogens and one carboxyl group, undergo remotion of peptide protons at very low pHs (<3). Deprotonation becomes more difficult passing to polymer **1** and then to **2**, also because of the inductive effect exerted by the methyl group inserted between peptide nitrogens, as suggested by the increased pH value where deprotonation takes place (Fig. 3).

By superimposing species distributions, evaluated from the corresponding log β's, it is possible to see the pH-range where the different complex species predominate. Fig. 3 shows the case of polymer **1**, where CuH(−1)L is present in higher amount and over a wider range of pH. In polymer **3** the interval of existence of this species is extended from 2 to 12, reaching a plateau with 90% of CuH(−1)L species between pH 5 and 9 where it is the most important species in solution. With the exception of the amphoteric polymer **3**, all the other basic polymers reveal closer stabilities for the CuH(−1)L species. This also occurs for polymer **5**, in spite of the presence of only one tertiary amino group, anchoring the metal ions. This polymer, in fact, is able to form

complex species by coordinating metal ions through the tertiary and deprotonated peptide nitrogens. The deprotonation of the latter is facilitated by the closeness of carboxyl group, which is also directly involved in the coordination process. The polydentate character of polymer **3** is consistent with its higher values of log β and λ_{max}. The values of thermodynamic parameters (Table 4) can be justified either by assuming a coordination only by the amino groups or by the involvement of the COO^- group.

The different $-\Delta H^\circ$ values estimated for the CuL and CuH(−1)L complexes, together with the markedly different values of ΔS° may suggest that these species have a different structure, as also confirmed by the spectroscopic data [17,24,31]. In all cases, the molar absorption coefficient (ε) was found sensibly lower than in PAA2 [23]. For the CuH(−1)L species, polymers **1** and **3** have ε values of 129 and 109 dm^3/mol cm, respectively, while the CuL species of polymer **1** has $\varepsilon = 98$. Such values are consistent with the complex species of less distorted octahedral geometry, since these species tend to possess lower ϵ values, presumably because of orbital mixing with higher symmetry fields [17,18,25].

The COO^- group is involved in coordination with the copper(II) ion to satisfy the stoichiometric ratio, as suggested by the ability to form complex species with calcium(II) ions through electrostatic interactions [7] and by viscometric titration data (Fig. 2). A comparison between the behaviour of reduced viscosity (η_{sp}/C) and the distribution curves shows that the minimum viscosity is reached when the CuH(−1)L formation is maximum. Beyond the minimum, viscosity increases again for the presence of uncomplexed COO^- groups, which induce higher electrostatic repulsion (see Section 2.2). In fact, if the Cu(II)/L molar ratio is increased from 0.5 to 1, a flatter viscometric titration curve is obtained, owing to the decreased amount of free carboxylate groups not coordinated to copper(II) ions. A slightly further increase of η_{sp}/C cannot be justified by the increase of the negatively charged CuH(−2)L species that start to form only beyond pH 8.

3. Conclusions

From the physico-chemical characterisations performed on the above basic and amphoteric PAAs that were synthesised from flexible bis-acrylamides (MBA, EBA, BAC) instead of the rigid 1,4-bis-acryloylpiperazine employed to prepare traditional PAAs, the following conclusions can be drawn.

1. Amido groups of flexible PAAs being closer, they have higher electron-withdrawing properties, resulting in polymers with lower basicity compared to the traditional PAAs. This also causes lower shielding and increased polyelectrolyte behaviour. In fact, an "apparent" behaviour of log K is observed upon protonation of the first basic site. Following this, a charged or neutral species is produced, which exhibits higher or lower shielding properties among the different repeating units, depending on the bis-acrylamide involved.
2. The complexing behaviour with copper(II) ions reveals the presence of three complex species. Besides the simple complex of CuL stoichiometry, which is formed at low pH involving only tertiary nitrogens in the coordination process, the most important species in solution is CuH(−1)L. This is formed through the deprotonation of neighbouring peptide nitrogens, which can be facilitated by the insertion of electron-withdrawing groups, such as COO^-, that induces higher stability and greater blue-shift of λ_{max}, even at low pH values.

The results of this paper may well render a contribution to the wide interest in protein-mediated biological processes involving metal ions [32–35]; the peptide residues is a relevant bonding site for the copper(II) ion in a number of metalloenzymes.

4. Experimental

4.1. Materials

Methylene-*N*,*N*′-bis(acrylamide) (MBA, 99% purity), triethylamine (TEA, 99%), HCl (0.1 M and 1 M) and NaOH pellets were purchased from Fluka Co., and used without further purification. Methylamine (MMA, 40% w/v aqueous solution), 2,2′-bis(acrylamido)acetic acid (BAC, 97%), *N*,*N*′-dimethylethylenediamine (DMEDA, 97%) and 4-methoxyphenol were purchased by Aldrich Co. The MMA solution was titrated potentiometrically with 1 M HCl and its experimental titre (40.8% w/v) was employed in polymerisations. BAC was recrystallised from methanol (10 ml per gram of BAC) while DMEDA was distilled under reduced pressure (0.1 Torr) just before use. *N*,*N*′-ethylidenebis(acrylamide) (EBA) was synthesised as reported in [36].

4.2. Synthesis

4.2.1. Preparation of *1*

MBA (7.00 g; 44.9 mmol) and 4-methoxyphenol (5 mg) were suspended in distilled water (30 ml); the mixture was cooled to 5°C and DMEDA (3.95 g; 44.9 mmol) was added under stirring and nitrogen stream. The mixture was left at 25°C, in the dark and under nitrogen atmosphere for 4 days. After addition of 1 M HCl up to pH 3, the resulting solution was diluted with distilled water, ultrafiltered at 10°C through a membrane with cut off 3000 and freeze-dried. Yield: 10.3 g. Potentiometric analysis: purity 97 wt%.

^{1}H NMR: $\delta = 2.21$ (3H, s, CH_3), 2.42 (2H, t, CO–CH_2), 2.53 (2H, s, CO–CH_2–CH_2–N), 2.71 (2H, t, N–CH_2–CH_2–N), 4.55 (1H, N–CH_2–N).

*4.2.2. Preparation of **2***

EBA–DMEDA (**2**) was prepared exactly under the same conditions as **1** by substituting EBA (7.50 g; 44.9 mmol) for MBA. Yield: 11.3 g. Potentiometric analysis: purity 92 wt%.

^{1}H NMR: $\delta = 1.38$ (3H, d, C–CH_3), 2.76 (4H, t, 2 × CH_2CO), 2.90 (6H, s, 2 × N–CH_3), 3.45 (4H, t, 2 × CO–CH$_2$CH_2–N), 3.60 (4H, s, 2 × N–CH_2–CH_2–N), 5.61 (1H, q, C*H*).

*4.2.3. Preparation of **3***

BAC (12.01 g; 60.6 mmol) and 4-methoxyphenol (10 mg) were dissolved in 3 M NaOH (20.2 ml); the solution was cooled up to 5°C and DMEDA (5.34 g; 60.6 mmol) was added under nitrogen stream and stirring. The mixture was left at 25°C, in the dark and under nitrogen atmosphere for 3 days. After addition of 1 M HCl up to pH 2, the resulting solution was ultrafiltered at 10°C through a membrane with cut off 3000 and freeze-dried. Yield: 17.1 g. Potentiometric analysis: purity 93 wt% (on the basis of protonated nitrogen), 85 wt% COOH form.

^{1}H NMR: $\delta = 2.89$ (2H, t, CH_2CO), 2.99 (3H, s, methyl), 3.57 (2H, t, COCH$_2$CH_2N), 3.75 (2H, s, N–CH_2CH_2–N), 5.68 (1H, s, C*H*).

*4.2.4. Preparation of **4***

MBA–MMA (**4**) was prepared under the same conditions as **1** by substituting MMA solution (3.42 g, $c = 40.8$ wt%, 44.9 mmol) for DMEDA. Yield: 5.1 g. Potentiometric analysis: purity 100 wt%.

*4.2.5. Preparation of **5***

BAC–MMA (**5**) was prepared under the same conditions as **3** by substituting MMA solution (4.61 g, $c = 40.8$ wt%, 60.6 mmol) for DMEDA. Yield: 5.2 g. Potentiometric analysis: purity 92 wt% (tertiary nitrogen), 10 wt% COOH form.

4.3. GPC measurements

Gel Permeation Chromatograms were obtained at 25°C making use of TSK-GEL G3000 PW and TSK-GEL G4000 PW columns connected in series, using Tris buffer pH 8.09 as mobile phase; sample concentration: 4 mg/ml; injection volume: 25 ml; loop size: 20 ml; flow rate: 1 ml/min (Knauer model HPLC Pump 64); column dimensions: 300 × 7.5 mm^2. The products were checked by a Knauer mod UV detector operating at 220 nm.

4.4. Spectroscopic measurements

Infrared spectra were run on a Jasco 5300 FT-IR spectrophotometer by casting from water ($c = 1$ wt%) on ZnSe windows.

Proton NMR spectra were recorded on a Varian XL 200 spectrometer with a solution freshly prepared by dissolving 10 mg of polymer in DMSO-d_6 or D_2O. Chemical shifts were measured by taking the residual DMSO peak as internal reference signal.

Electronic (UV–Visible) spectra of copper(II)–polymer solutions were recorded at different pHs on a Pharmacia LKB-Biochrom 4060 spectrophotometer using 1 cm silica cells. Stepwise forward (with NaOH 0.1 M) and backward (with HCl 0.1 M) titrations by changing the pH of the solution (100 ml, 0.1 M NaCl) containing the appropriate polymer (ca. 0.2 mmol) and copper(II) ions (L/Cu(II) molar ratios from 1 to 3 were carried out at 25°C. The ϵ value was calculated on the basis of the species distribution obtained by the previously reported program FIT [16].

4.5. Viscometric measurements

Intrinsic viscosity and viscometric titration data were measured at 25°C with an AVS 310 Schott–Gerate viscometer. Freshly prepared solutions (25 ml) in 0.1 M NaCl, containing a weighed amount of polymer (ca. 0.2 mmol) and a measured quantity of HCl standard solution were stepwise titrated with a standardised 0.1 M NaOH solution delivered by a Metrohm Multidosimat piston burette. Titrations in the presence of metal(II) ions were obtained in a similar manner on solutions containing a known quantity of Cu(II) ions to have a molar ratios L/Cu(II) of 1 and 2. The species distribution was calculated by means of the program FIT.

4.6. Potentiometric measurements

The potentiometric apparatus has been described previously [18]. Titrations were performed at a constant temperature (25°C) in 0.1 M NaCl with a digital PHM-84 Radiometer potentiometer, equipped with a pHG211 High pH Glass Electrode (Radiometer) and a Ref201 Reference Electrode (Radiometer), and a Metrohm Multidosimat piston burette connected to a computer (Olivetti M20) controlling and automatically recording potentiometric data (e.m.f. readings (mV) in relation to volume (ml) of added titrant).

A weighed amount (0.1–0.3 mmol) of solid polymer, in the hydrochloride form, was dissolved in the thermostatted glass cell containing 100 ml of 0.1 M NaCl, under magnetic stirring. A measured volume of standardised 0.1 M HCl was added to the polymer solution to reach a low pH. Titrations with 0.1 M NaOH and back-titrations with 0.1 M HCl were performed under a pre-saturated nitrogen stream flowing over the surface of the solution to avoid contamination of CO_2 from the outside atmosphere. The equilibrium condition was reached within the 300 s we programmed for each step of titrant addition. Both forward and backward titrations were found in reliable results. From three to five replicates of each polymer at different concentrations confirmed a good agreement.

Basicity constants were evaluated by means of the APPARK program [16] on an Olivetti M20 computer. Potentiometric data were also processed with the program

SUPERQUAD [28] on a Macintosh LCII computer and the results of log *K*s were in close agreement.

The complexation study with copper(II) ions was studied in a similar way by using three different L/Cu(II) molar ratios (1, 2 and 3). The stability constants were evaluated with the SUPERQUAD program.

4.7. Calorimetric measurements

The titration calorimetry (Tronac, mod 1240) has been described previously [24]. Continuous calorimetric titrations were performed in the Isothermal mode at 25°C with a water-bath controlled by a PTC-40 (Precision Temperature Controller, from Tronac, Inc.). The 25-ml stainless-steel reaction vessel, filled with 0.1 M NaCl and containing a weighed quantity of polymer (0.1–0.2 mmol) and a measured volume of standard 0.1 M HCl, was titrated with standardised 0.1 M NaOH at a constant BDR (burette delivery rate) of 0.1000 ml/min delivered by the Gilmont burette. Either forward and backward titrations with NaOH and HCl solutions confirmed reliable data. Calibration of the instrument was made with Tris–HCl and HCl–NaOH titrations before each run. All the calorimetric titration data (heat (cal) in relation to volume (ml) of titrant added) were stored on a floppy disk for processing.

The enthalpy changes ($-\Delta H^\circ$) were evaluated by the program FIT reported previously [16] and the ΔS° calculated combining the $-\Delta G^\circ$ (from the basicity constant, $-\Delta G^\circ = RT \ln K$) and $-\Delta H^\circ$ obtained at 25°C. Five or more replicates were in good agreement and averaged.

For the polymer–copper(II) complexes, the heats of complex formation were obtained by titrating the polymer/metal(II) (with molar ratios of 1, 2 and 3) acidified solutions with 0.1 M NaOH. Back-titrations with 0.1 M HCl confirmed a good agreement. The $-\Delta H^\circ$ for complex formation species was computed by the program FIT running on the Olivetti M24 computer.

In all cases, the experimental procedure, including the chemistry and calibration heater, was controlled automatically by the ISOTHERMAL program (from Tronac, Inc.) on a North Star CCP 930 computer connected to the instrument [24].

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