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Coordination Chemistry Reviews
190–192 (1999) 683–706

COORDINATION CHEMISTRY REVIEWS

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Porphyrin assemblies as chemical sensors

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Accepted 13 March 1999

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PII: S0010-8545(99)00106-X

Abstract

A simple 'non-covalent' approach was employed to obtain sensitive and specific sensors for pH, DNA and metal ions, by 'mixing' commercially available chemicals such as cationic or anionic water-soluble porphyrins and polypeptides. For example, under the appropriate conditions, the supramolecular complex formed between the anionic *meso*-tetrakis(4-sulfonatophenyl)porphine (H_2TPPS) and the protonated form of poly-Lysine can behave as pH-sensor. In fact, H_2TPPS in the pH range 5.5–12 exists in a monomeric form, and its fluorescence is not pH-dependent. However, at low pH values (≤ 7), the protonated poly-Lysine promotes porphyrins binding and self-aggregation with consequent strong quenching of their fluorescence, while at pH values higher than 9–10, the porphyrins exist in solution essentially as free monomers and are characterized by an intense fluorescence emission. As a consequence, the H_2TPPS fluorescence intensity versus pH behavior shows a sigmoidal profile. Interestingly, the molecular recognition processes leading to the formation of these aggregates can be also modulated by using matrices of different nature and length as well as employing porphyrins containing different central metal ions with particular coordination geometries. In such a way we have been able to develop a whole family of sensors covering a wide range of pH. These supramolecular aggregates can also be employed as sensors for DNA. In fact, the addition of DNA (which is a poly-anion) to a preformed H_2TPPS/poly-Lysine system (pH ≤ 7), causes a displacement of the porphyrin bound to the poly-cationic matrix with consequent increase in the fluorescence intensity of the solution. Therefore, since the variation in fluorescence emission is linearly related to the concentration of DNA added, we have employed such supramolecular system to develop a simple and rapid method for the quantitative determination of DNA in solution. Finally, a remarkable acceleration of the insertion of copper(II) and zinc(II) in cationic porphyrins is observed when these porphyrins are monodispersed on the surface of negatively charged matrices, such as anionic poly-Glutamate. Such peculiarity allowed us to develop a specific fluorescent sensor for both metal ions capable of detecting their presence even at very low concentrations in the nanomolar range.

Keywords: Porphyrins; Molecular recognition; Supramolecular chemistry; Sensors; Resonance light scattering; Fluorescence spectroscopy

1. Introduction

Molecular recognition is one of the most elegant and powerful driving force that Nature has chosen for life machinery to work. It regards not only the specificity of a given interaction, but, most importantly, it represents a simple and reproducible way to store information which can be released in a single reaction (e.g. the catalytic action of an enzyme) or hold along a cascade of biochemical events, the utmost example being the central 'dogma' of molecular genetic (replication → transcription → translation). Such specificity is controlled and fine tuned by weak intermolecular (or non-covalent) interactions and, therefore, it is based on a high degree of complementarity between the species involved, not only in shape and size but also in their electronic properties (i.e. charge distribution).

It is no wonder, therefore, that such fashionable and intriguing features of Nature have attracted the interest of many researchers who carefully observe

natural systems to get suggestions for biomedical, analytical or technological applications. In particular, the possibility of releasing 'stored' information upon binding of specific species is the key point for the branch of chemistry interested to sensors. Additionally, this particular research field needs the transduction of the recognition event to an external signal (optical, electrical, etc.).

One of the most used and simple ways of signal transduction is obtained by monitoring fluorescence. There are various advantages in using fluorescence; (i) it is a powerful visual phenomenon; (ii) its sensitivity is several orders of magnitude higher than that of absorption spectroscopy; (iii) a spectrofluorimeter is a piece of equipment commonly available in many scientific laboratories. As a consequence, a large number of fluorescent sensors for pH or metal ions have recently been designed and synthesized by means of supramolecular 'covalent' approaches [1–5], most of them being two-component systems in which a 'receptor' unit is covalently linked to a 'sensing' one.

However, a supramolecular photochemical species can be also seen as a chemical complex in which one or more photoactive component(s) is (are) assembled together by and with an 'organizing receptor' [6]. The properties of such species are 'modulated by the arrangement of the bound (photoactive) units as determined by the organizing receptor' [6], i.e. there is a strong interplay between the properties of the photochemical supramolecular species and the molecular recognition processes which lead to its formation. Therefore, the knowledge of these processes and a reasonable prediction of the photophysical variations of the photoactive component(s) upon assembly should allow the design of complex species characterized by new properties.

We followed the latter approach to design a series of sensors by means of a 'non-covalent' strategy which allowed us to achieve specific photochemical sensors. Owing to their high extinction coefficients and tunable fluorescence emission (e.g. by changing the central metal ion), we have selected porphyrins as good sensing units. Furthermore, the introduction of polar groups at the periphery of the porphine ring makes them water-soluble and allows the formation of 'non-covalent' supramolecular species onto ionizable polymeric templates which can act as organizing receptors when they are oppositely charged [7]. In the following examples we use, as matrices, polypeptides bearing ionizable groups in the side chains, as poly-Lysine and poly-Glutamate. The use of such matrices allows to tune both porphyrin-matrix interactions and porphyrin aggregation state: (i) by modulating with pH the number of charges; (ii) by choosing matrices of different nature and length. Also, by properly modifying the experimental conditions, it is possible to tune the macroscopic properties (absorption, emission features, etc.) of the supramolecular complexes.

On the other hand, the formation of these supramolecular complexes depends upon several factors (such as pH, ionic strength, etc.) and consequently they are less 'robust' than those obtained by the classical 'covalent' approach. Finally, we would like to underline that in the following context the term sensor is not used to indicate a device, but rather a chemical system able to recognize a species and to report its recognition (e.g. by spectroscopic variations).

As will be discussed, using such simple 'non-covalent' approach on commonly available chemicals such as cationic or anionic water-soluble porphyrins and polypeptides, several sensitive and specific sensors for pH, DNA and metal ions have been developed.

2. Porphyrin supramolecular complexes as fluorescent pH sensors

This project begun from the observation that anionic porphyrins interaction with poly-Lysine, may lead, under appropriate conditions, to the formation of aggregates [8–11]. The very first recognition processes are primarily driven by electrostatic interactions: those between the negatively charged peripheral groups of the porphyrins and the protonated side chains of the matrix and those among porphyrins themselves [12–15]. In fact, once a large enough porphyrin 'critical' concentration onto the polymer matrix is reached, van der Waals and solvophobic forces may induce porphyrin aggregation which, in many cases, leads both to remarkable absorption hypochromicity and fluorescence quenching [16,17].

In this context, we have shown [10,11] that it is possible to control porphyrin fluorescence by modulating, through pH variations, the interaction between the anionic porphyrins (the *photoactive components*) and the protonated poly-Lysine (the *organizing receptor*). This way, such systems can be employed to develop a whole family of supramolecular fluorescent 'pH-sensors' [10]. Also, as will be shown later, the interval of highest sensitivity can be easily modulated over a wide pH range by simply selecting the proper chemical components (free porphyrins with specific central metal ions, and poly-Lysine of appropriate length) and experimental conditions (poly-Lysine/porphyrin ratio, ionic strength).

2.1. Spectroscopic characterization of H_2TPPS/poly-Lysine system

One of the porphyrins selected for our study is the *meso*-tetrakis(4-sulfona-

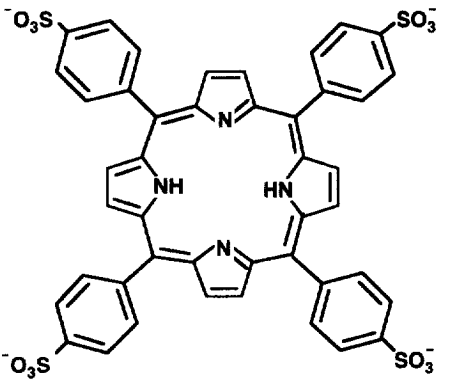

Fig. 1. Molecular structure of the *meso*-tetrakis(4-sulfonatophenyl)porphine (H_2TPPS).

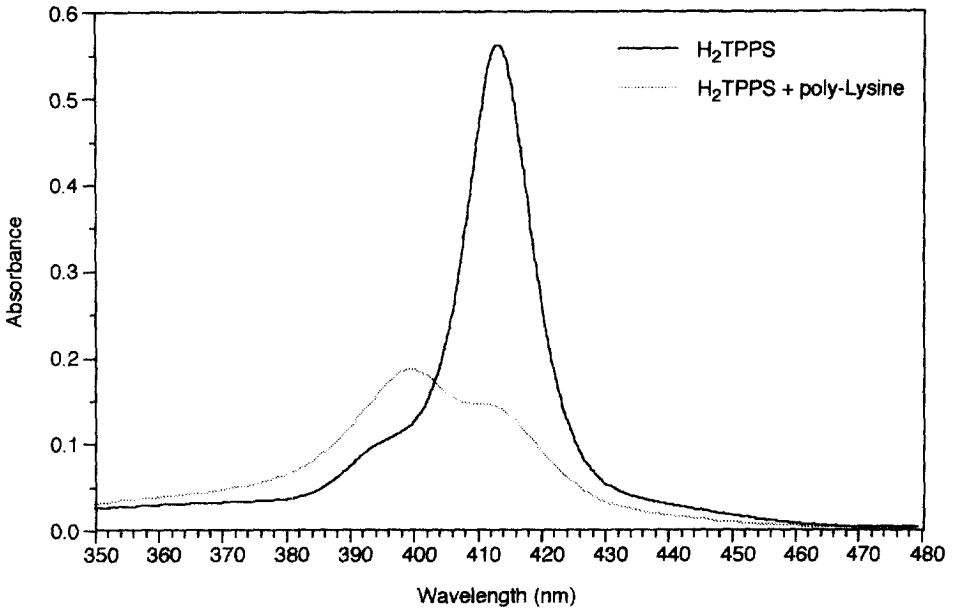

Fig. 2. Absorption spectra of H_2TPPS (1×10^{-6} M) in the absence (—) and in the presence (...) of poly-Lysine (1.3×10^{-5} M). Modified from Ref. [40] with kind permission of Elsevier Science.

tophenyl)porphine (H_2TPPS) (Fig. 1) and our investigation started by characterizing spectroscopically the behavior of H_2TPPS in aqueous solution both in the absence and in the presence of poly-Lysine. H_2TPPS porphyrin is characterized by a very high absorption extinction coefficient ($\varepsilon_{414} = 5{,}33 \times 10^5$ M^{-1} cm^{-1}) [18] and a good fluorescence quantum yield ($\phi = 0.08$) [19]. Under the experimental conditions employed ([H_2TPPS] = 1 μM, pH range 5–12) the tetranionic porphyrin is essentially in the monomeric form. However, as shown by the absorption spectra reported in Fig. 2, upon addition of poly-Lysine the Soret band of the free-porphyrin ($\lambda_{max} = 414$ nm) experiences a strong hypochromic effect ($\approx 60\%$) and some broadening. The remarkable spectral changes observed upon the addition of poly-Lysine are clearly indicating the formation of new species. In particular, similar variations are commonly attributed to porphyrins aggregation processes [20]. In Fig. 3 is reported the fluorescence emission spectrum of the H_2TPPS/poly-Lysine system under the same experimental conditions. Here, the interaction between porphyrin and poly-Lysine is characterized by a strong fluorescence quenching respect to the radiation emission of the porphyrin free in solution. Such spectral variations are, as well, typical of porphyrin transitions from monomeric to aggregated form [16,17,21–23]. A further strong evidence of the formation of large supramolecular aggregates was provided by resonance light scattering (RLS) measurements [24]. As shown in Fig. 4, the RLS spectrum of the free monomeric porphyrin does not show any detectable signal above background. On the contrary,

the RLS spectrum of a solution of H_2TPPS containing poly-Lysine is characterized by a strong large band in the Soret region centered at about 405 nm, indicating the formation of extended supramolecular species capable of scattering light. Moreover, the blue-shift of the Soret band, in the presence of the cationic matrix, suggests the reciprocal disposition of the aggregated porphyrins to be face-to-face (H-type aggregates) [25,26].

2.2. H_2TPPS/poly-Lysine aggregates as pH-sensors

Spectrofluorimetric pH-titrations show that, the fluorescence intensity of the free H_2TPPS is not pH dependent in the range 5.5–11.5. On the other hand, in the presence of poly-Lysine, the profile of the fluorescence intensity versus pH shows a sigmoidal shape (Fig. 5). Back-titrations with HCl show that these aggregation processes are fully reversible. Therefore, it turns out that such sigmoidal shape must be due to the equilibrium between aggregated and free anionic porphyrins. Here, fluorescence variations report only the porphyrins aggregation state, and the pH dependence of the equilibrium indicates that the formation of the supramolecular species is triggered by the presence of positively charged amino groups of poly-Lysine. Thus, at low pH values, where poly-Lysine is extensively protonated, the equilibrium is shifted toward binding and concomitant aggregation (strong fluorescence quenching in Fig. 5). Here, only the bound porphyrins in aggregated form

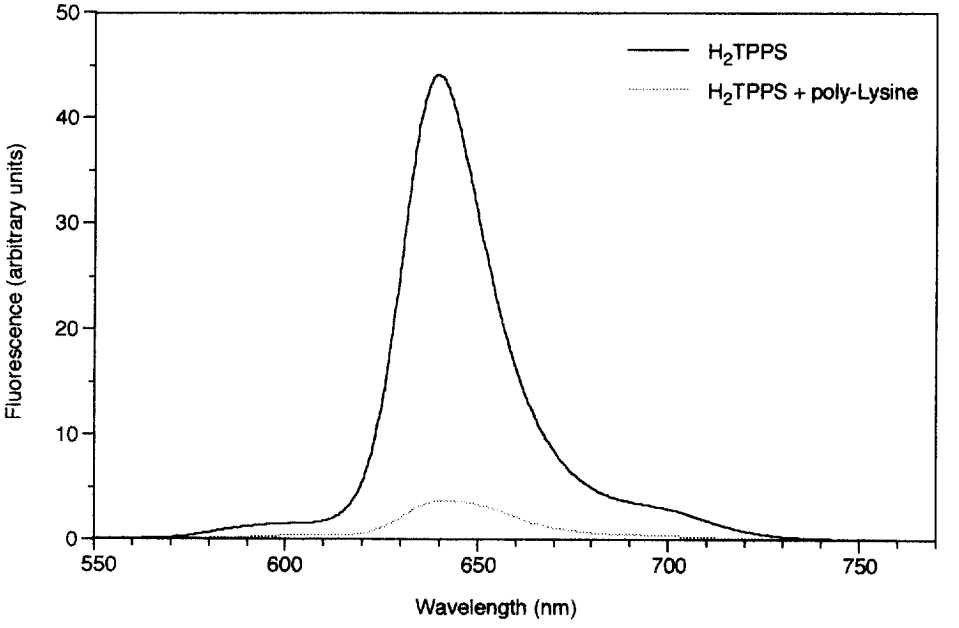

Fig. 3. Fluorescence emission spectra of 1×10^{-6} M H_2TPPS in the absence (—) and in the presence (...) of poly-Lysine (1.3×10^{-5} M). $\lambda_{exc} = 414$ nm. Modified from Ref. [40] with kind permission of Elsevier Science.

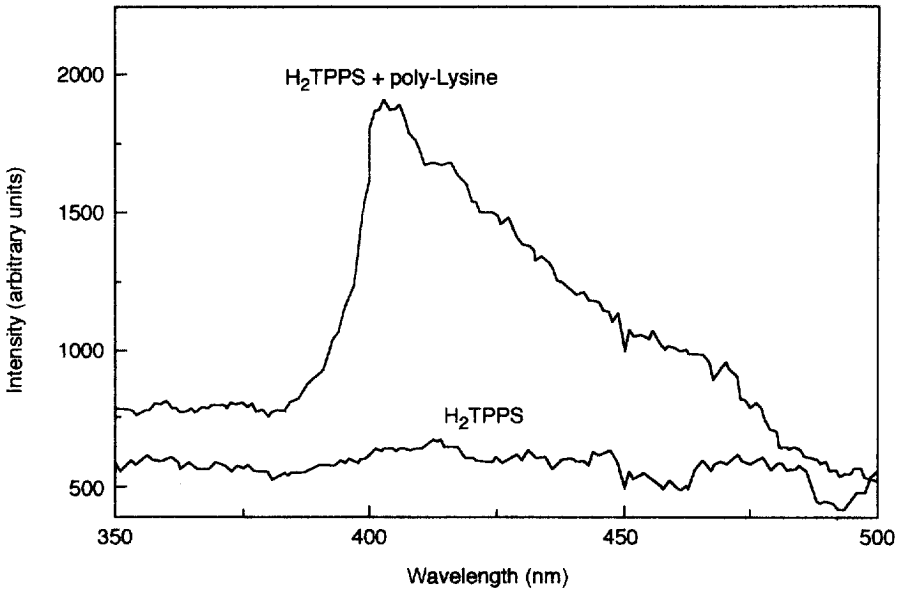

Fig. 4. Resonance light scattering (RLS) spectrum of 1×10^{-6} M H_2TPPS in the absence and presence of 1×10^{-4} M poly-Lysine. Modified from Ref. [40] with kind permission of Elsevier Science.

contribute to the emission quenching. At higher pHs the porphyrins tend to dissociate from the poly-Lysine matrix and eventually exist as monomers in solution (high fluorescence emission in Fig. 5). Therefore, it is possible to

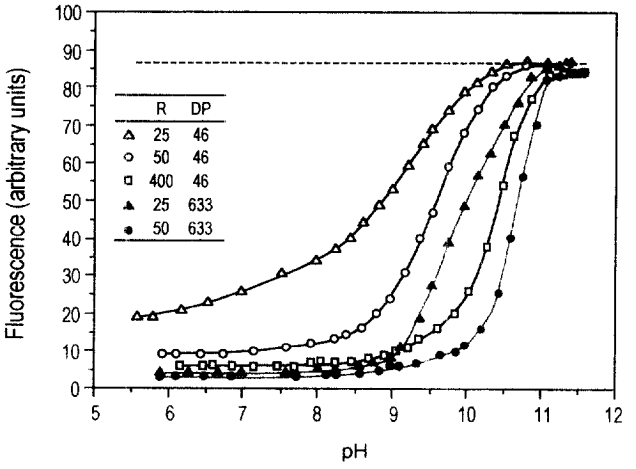

Fig. 5. pH dependence of the fluorescence emission ($\lambda_{ex} = 405$ nm, $\lambda_{em} = 640$ nm) of H_2TPPS (1×10^{-6} M) in absence (---) and in the presence (——) of poly-Lysine. *R* is the [poly-Lysine]/[H_2TPPS] ratio. DP is the poly-Lysine degree of polymerization. Modified from Refs. [10,11] with kind permission of Kluwer Academic Publishers and the American Chemical Society.

switch the fluorescence emission on and off by simply changing the pH by about 2 units.

Taking a closer look at the equilibria involved in these aggregation processes it turns out that a quantitative characterization of each equilibrium step becomes quite complex. In fact, we are dealing with multiple equilibria which can be represented as follows:

$$\begin{array}{c} \text{poly-Lysine} + n\,\text{H}^+ \\ \Big\downarrow\!\!\uparrow \\ m\,\text{Porphyrin} + \text{poly-Lysine(H}^+)_n \quad \rightleftharpoons \quad (\text{Porphyrin})_m\text{•poly-Lysine(H}^+)_n \end{array} \qquad (1)$$

where n is the number of protonated lysine residues per strand and m represents the number of aggregated porphyrins per poly-Lysine strand. Obviously, to different values of n correspond different charge densities on the polymer which, therefore, will affect the number of bound porphyrins (m). It follows that for any values of n and m it pertains the formation of different species and, therefore, a different equilibrium constant for the formation of the supramolecular complexes.

2.3. Influence of poly-Lysine concentration and chain length

Interestingly, the formation of these supramolecular species is strongly affected by the matrix concentration and length. As reported in Fig. 5, the fluorescence intensity at pH < 8 decreases drastically increasing the poly-Lysine/H_2TPPS ratio (R) from 25 to 50 and then levels off. Also, RLS spectra (data not shown) obtained at $R = 25$ are more intense than those measured at $R = 50$, while at $R = 100$ no signal is detectable, owing, most likely, to the presence of very small aggregates. In fact, this trend cannot be ascribed to a lower extent of porphyrins aggregation going from $R = 25$ to $R = 100$, because the spectrofluorimetric data show the opposite one, i.e. the concentration of aggregated porphyrins increases on increasing R values.

These observations indicate that the concentration of the total bound porphyrin in the aggregated form increases with poly-Lysine concentration, but H_2TPPS aggregates become smaller and smaller. These two effects can be easily explained by a larger availability of binding sites. Also, going from $R = 25$ to $R = 100$ the inflection point of the various curves is shifted towards higher pHs (Fig. 5). No further shift is observed for R values higher than 400.

Clearly, the considerations about the multiple co-existing equilibria (Eq. (1)) hold for each of the curves of Fig. 5. However, as shown by RLS data, at a given poly-Lysine concentration pertains an initial value of m (the number of aggregated porphyrins per strand) which differs from those of the other curves and leads to a series of subsequent equilibria (Eq. (1)) which are function of R. Furthermore,

Table 1
Apparent pK for the supramolecular complexes formed by H_2TPPS and ZnTPPS with poly-Lysine (degrees of polymerization, 46 and 633).

R	pK for H_2TPPS/poly-Lysine (DP = 46)	pK for H_2TPPS/poly-Lysine (DP = 633)	pK for ZnTPPS/poly-Lysine (DP = 46)
25	9.0	9.8	10.0
50	9.5	10.6	–
100	9.9	–	10.6
400	10.4	–	–

other effects than the poly-Lysine/porphyrin ratio may modulate the equilibria (Eq. (1)). For instance, using longer poly-Lysine strands (Fig. 5) we found both at $R = 25$ and 50 not only a stronger fluorescence quenching but also a shift of the 'inflection' point with respect to the analogous curves obtained using shorter poly-Lysine strands (Fig. 5). This result is probably related to stronger electrostatic fields in longer chains polymers. This indicates that the shift of the inflection point, observed by varying R, cannot be simply related to a shift toward the right side of the equilibria (Eq. (1)) for concentration effects but rather reflects the variation in population distribution of aggregated porphyrins (dimers, trimers...) on varying the number of available binding sites.

However, despite this complexity, the fully reversible behavior of the single fluorescence titration curves suggests that we can treat each curve as a whole system of subsequent equilibria which describes the dissolution of a given initial supramolecular assembly, where each system (i.e. each curve) 'melts' at a different pH, which depends on R and on the polylysine length. Then, a reasonable approach to simplify the multiple equilibria (Eq. (1)) consists in considering a single comprehensive equilibrium for porphyrin binding, where poly-Lysine does not appear explicitly:

$$\text{Free porphyrins} \overset{+\text{H}^+}{\underset{-\text{H}^+}{\rightleftarrows}} \text{Aggregated porphyrins} \qquad (2)$$

The pH dependence of the equilibrium (Eq. (2)) allowed us to associate to it an apparent pK which simply indicates the pH at which the concentrations of aggregated and free porphyrins are equal, independently of the m values. The various pK values can be graphically deducted and are reported in Table 1. As already discussed, the shift of the inflection points, observed by increasing R or varying the polymeric strand length, reflects a different 'stability' to pH changes of various supramolecular porphyrin aggregates and this effect can be exploited to develop supramolecular fluorescent sensors covering a wide range of pH. In particular, it looks like the smaller the aggregate the higher is the resistance of the system to pH variations.

2.4. *ZnTPPS/poly-Lysine and SnTPPS/poly-Lysine systems*

We have also investigated [11] the behavior of similar systems where the penta- and hexa-coordinated ZnTPPS and SnTPPS were employed [27]. In particular, the

spectroscopic features of ZnTPPS in the presence of the polypeptide are very similar to those previously described for the parent free-porphyrin. In fact, following the addition of poly-Lysine ($R = 25$) the absorption spectra show a strong hypochromicity (about 60%) and broadening of the Soret band. Because of the presence of one axial ligand [27], we have hypothesized [11] that ZnTPPS self-aggregation is limited to the formation of face-to-face dimers.

The spectrofluorimetric titrations of the ZnTPPS/poly-Lysine system (Fig. 6) are similar to those already described for H_2TPPS. However, as reported in Table 1, at $R = 25$, the ZnTPPS supramolecular complex is about as stable as that formed by H_2TPPS at $R = 100$, the pK values being 10.0. Again, these observations suggest that to small aggregates pertain a series of subsequent equilibria which leads to high pK values. Finally, between pH 7 and pH 8 we observed an additional fluorescence inflection in the ZnTPPS/poly-Lysine titrations which we have tentatively assigned [11] to the deprotonation of the axially bound water to the metal site [4]. This inflection is not present in the absence of poly-Lysine (Fig. 6) and, therefore, must be related to aggregation processes.

Finally, as expected, a different behavior was observed for the hexa-coordinated Sn^{IV}TPPS [27]. This metallo-porphyrin was selected because the presence of two axial ligands prevents self-aggregation. The absorption spectra show that upon the addition of poly-Lysine ($R = 100$) the Soret band of this metallo-porphyrin is red-shifted by only 2 nm and its intensity decreases by about 40%, suggesting that porphyrins are essentially monomeric. Accordingly, as shown in Fig. 7, Sn^{IV}TPPS fluorescence is only slightly quenched (less than 10%) in the presence of the protonated polypeptide (pH < 8), confirming that the quenching observed for the

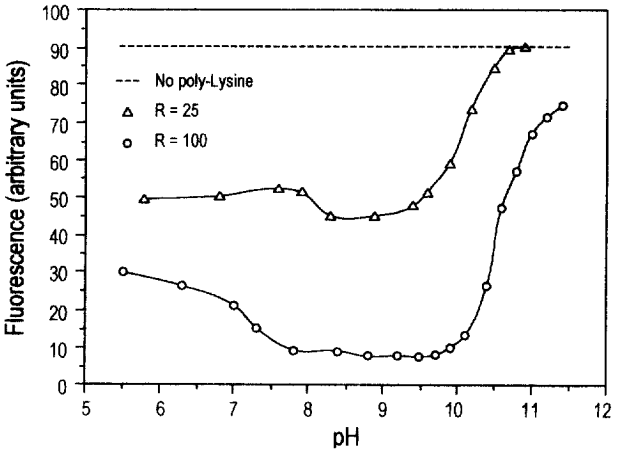

Fig. 6. pH dependence of the fluorescence emission of ZnTPPS ($\lambda_{ex} = 429$ nm, $\lambda_{em} = 604$ nm) in the presence (—) and in the absence (---) of poly-Lysine (polymerization degree = 46). Reprinted from Ref. [11] with kind permission the American Chemical Society.

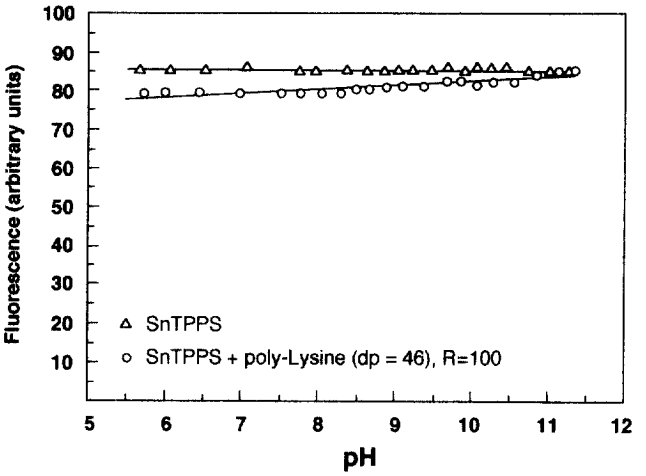

Fig. 7. pH dependence of the fluorescence emission ($\lambda_{ex} = 420$ nm, $\lambda_{em} = 600$ nm) of 1×10^{-6} M SnTPPS free in solution (△) and in the presence of 1×10^{-4} M poly-Lysine (○). Modified from Ref. [11] with kind permission the American Chemical Society.

H_2TPPS/poly-Lysine and ZnTPPS/poly-Lysine systems must be mainly due to short-range porphyrin-porphyrin interactions, as those occurring in self-assembled structures.

3. Porphyrin aggregates for quantitative DNA determination

Rapid and accurate determination of DNA concentration is important for many molecular biology applications such as gene mapping, molecular cloning, automated DNA sequencing and electrophoretic analysis. Yet, there are only few useful techniques available to accomplish both speed and accuracy at the same time. The most commonly used method in biochemical and molecular biology laboratories is a simple spectrophotometric determination [28] based on the strong absorption in the UV region (250–280 nm) of the purinic and pirimidinic bases of nucleic acids. The exact absorption wavelength and extinction coefficient depend on the base composition of the polymer. The detection limit of this method is around 1×10^{-6} M (mol of base pairs l^{-1}). When lower DNA concentrations need to be measured or the sample is contaminated by other components which absorb in the UV, several fluorescent methods can be used. Most of them take advantage of the enhancement in fluorescence emission of a small dye molecule upon binding to DNA. Since the fluorescence intensity is proportional to the mass of the nucleic acid, DNA concentration can be measured comparing the fluorescence emission of the sample relative to a standard. Several dyes can be used, such as Ethidium Bromide [28,29], DAPI (4′,6-diamidino-2phenyl indole) [30] and Hoechst 33258 [31–33] as well as other new fluorescent probes recently synthesized [34–37]. Such

methods are, in general, more sensitive and DNA concentrations of the order of 1×10^{-8} M or lower can be measured. Finally, other methods have been recently reported, based either on the increase of the resonance light scattering (RLS) signal of the α,β,γ,δ-tetrakis[4-(trimethyl-ammonium-yl)phenyl]porphine (TAPP) [38] or on the enhancement of room temperature phosphorescence (RTP) of a palladium/porphine complex upon interaction with DNA [39].

In this paragraph we describe how the H_2TPPS/poly-Lysine supramolecular system can be employed for quantitative determination of DNA in solution. Using a simple 'competition' method, DNA concentrations of unknown samples in the range 4×10^{-8} to 1.5×10^{-6} M can be rapidly and accurately measured [40].

3.1. DNA quantification by competition with H_2TPPS aggregated onto poly-Lysine

Our approach is based on a very simple idea. As described earlier, a supramolecular aggregate of the anionic H_2TPPS porphyrin (Fig. 1) is formed on the positively charged protonated poly-Lysine ($pK_a \approx 9.9$), where porphyrin fluorescence emission is almost completely quenched (Fig. 3). If DNA is added to such a pre-formed H_2TPPS/poly-Lysine supramolecular complex, the porphyrin becomes progressively replaced by the nucleic acid on the poly-Lysine surface, as schematically shown in

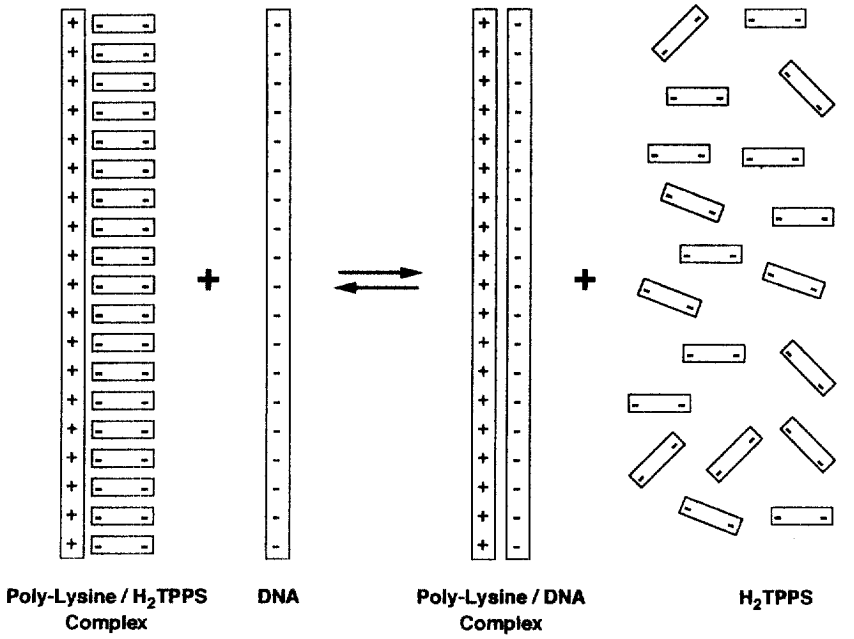

Fig. 8. Schematic representation of H_2TPPS displacement from the poly-Lysine surface upon addition of DNA. Reprinted from Ref. [40] with kind permission of Elsevier Science.

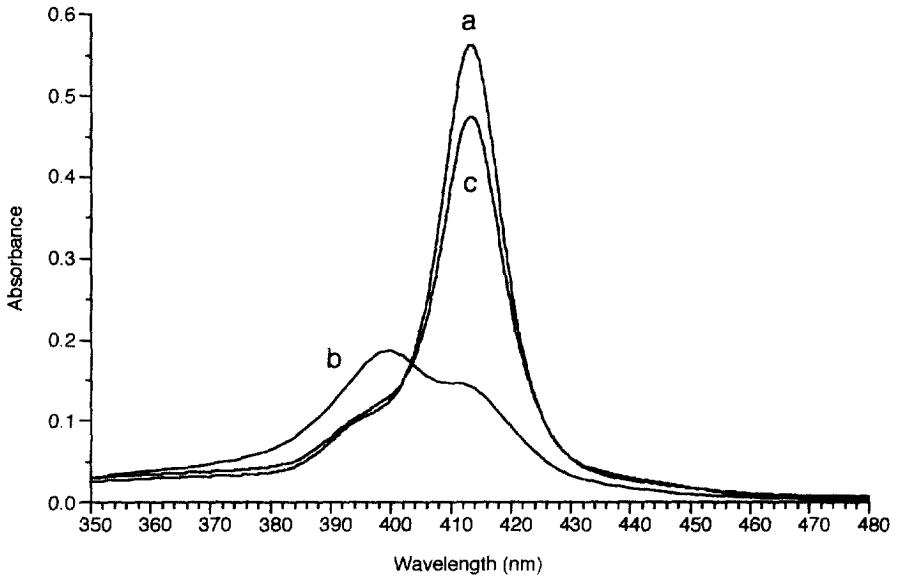

Fig. 9. Absorption spectra of 1×10^{-6} M H_2TPPS: (a) free in solution; (b) in the presence of 1.3×10^{-5} M poly-Lysine and (c) with poly-Lysine after the addition of 2×10^{-6} M DNA. Reprinted from Ref. [40] with kind permission of Elsevier Science.

Fig. 8. The displacement of the anionic H_2TPPS bound to the polymeric matrix by the highly charged DNA poly-anion results in an increased amount of monomeric porphyrin in solution, as shown by the absorption and fluorescence data reported in Fig. 9 (curve c) and Fig. 10 (curve c). Moreover, as shown in Fig. 11, the increase in fluorescence intensity at 640 nm (H_2TPPS emission maximum) turns out to be linearly dependent on the amount of DNA added. Therefore, by using DNA samples of known concentrations, it would be possible to determine the equation of the straight line which best fits the data obtained. Such equation could then be used as calibration for the determination of DNA concentrations in unknown samples.

Unfortunately, it turns out that it is quite difficult to find experimental conditions which give reproducible values of initial fluorescence intensity (in the absence of DNA) and of angular coefficients of the calibration lines. In fact, these supramolecular aggregates most likely do not form 'single species' but grow in one or more dimensions on the polymeric matrix, assembling in several structures different in organization and size. Therefore, due to their intrinsic nature these supramolecular aggregates can be characterized by slightly different quenching of the porphyrin fluorescence emission as well as by small variations in their response to the addition of DNA.

This drawback can be easily overcome by using the same solution H_2TPPS/poly-Lysine for all the steps of the quantification procedure. As shown in Fig. 12, the initial fluorescence intensity of such solution is measured, as well as after each addition of small volumes of a DNA solution of known concentration. Then,

successive aliquots of the same volume from a DNA sample of unknown concentration are added and the fluorescence intensity measured at each point. The two sets of data are fit with straight lines and their relative equations determined as:

$$\text{known} \quad \text{DNA concentration} \rightarrow y = k + mx \tag{3}$$

$$\text{unknown} \quad \text{DNA concentration} \rightarrow y' = k' + m'x \tag{4}$$

where y and y' represent the fluorescence intensity measured after the addition of each aliquot of, respectively, known DNA solution and unknown DNA solution, k, k' and m, m' are the intercepts and slopes of the two straight lines, x is the total volume added. At this point it becomes straightforward to correlate the angular coefficients of the two lines with the concentrations of the two DNA solutions c and c':

$$m:c = m':c' \tag{5}$$

and to determine the concentration of the unknown DNA solution c' as:

$$c' = (m'/m)c \tag{6}$$

Also, under the experimental conditions used, the system responds linearly for DNA concentrations up to about 1.5×10^{-6}. Above this limit the fluorescence intensity levels off to a constant value (corresponding to the emission of H_2TPPS

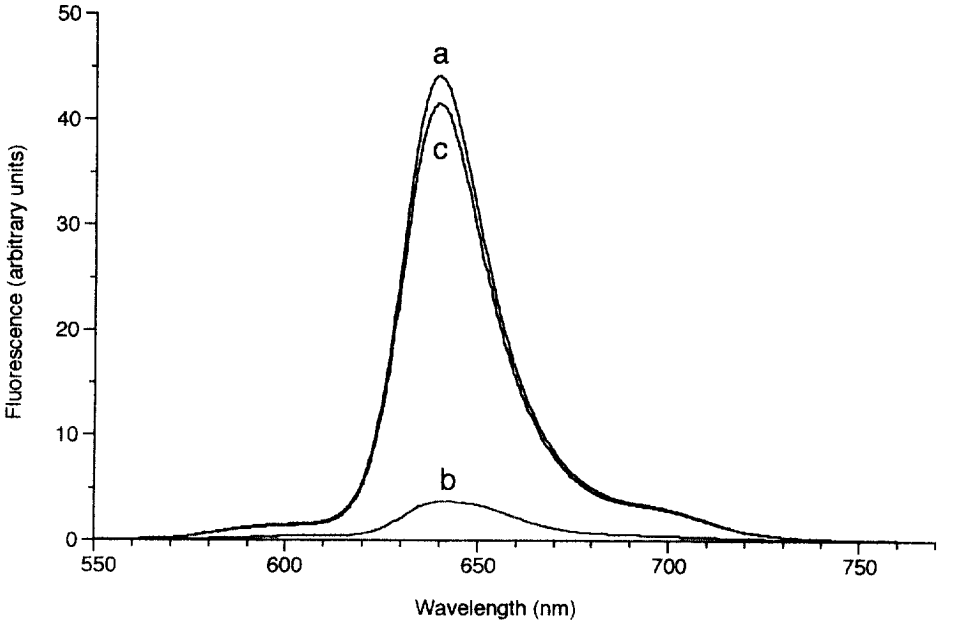

Fig. 10. Fluorescence emission spectrum of 1×10^{-6} M H_2TPPS: (a) free in solution; (b) in the presence of 1.3×10^{-5} M poly-Lysine and (c) with poly-Lysine after the addition of 2×10^{-6} M DNA. $\lambda_{exc} = 414$ nm. Reprinted from Ref. [40] with kind permission of Elsevier Science.

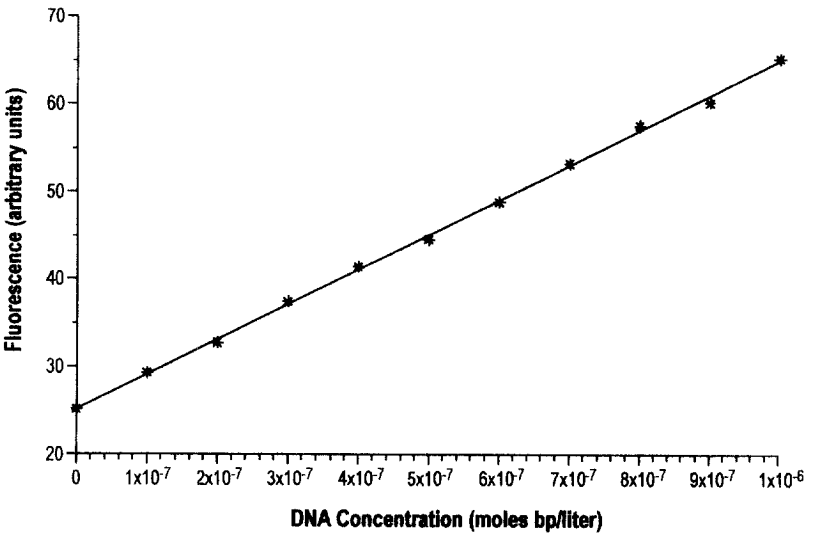

Fig. 11. Fluorescence intensity of the H_2TPPS/poly-Lysine system vs. DNA concentration (expressed in mol of base pairs l^{-1}). H_2TPPS = 1×10^{-6} M. Poly-Lysine = 1.3×10^{-5} M. $\lambda_{exc} = 414$ nm. $\lambda_{em} = 640$ nm. Reprinted from Ref. [40] with kind permission of Elsevier Science.

free in solution) and further addition of DNA does not produce any increase in fluorescence intensity. This occurs after complete displacement of all porphyrin molecules and concomitant saturation of the polymeric matrix by the DNA. Finally, the relative standard deviation (indicated with σ) was determined for every DNA quantitation experiment. The largest value of σ calculated correspond to a DNA concentration of 1.3×10^{-8} M. Therefore, imposing 3σ as lower limit of sensitivity of the method, the lowest detectable DNA concentration turns out to be around 4×10^{-8} M. Actually, the 'real' sensitivity is slightly higher since reasonably lower values of σ are commonly observed. Such limit results comparable with other DNA quantitation methods previously described.

3.2. Factors influencing the formation of H_2TPPS/poly-Lysine complexes and their effect on DNA quantification

Several are the factors which affect the formation of the H_2TPPS aggregates on poly-Lysine and, as a consequence, their behaviour in the DNA quantification assay.

The most important parameter is the total H_2TPPS concentration which needs to be held constant at around 10^{-6} M. In fact, at lower concentrations most of the porphyrin remains in monomeric form in solution and only limited aggregation could be observed. This leads to reduced variations in fluorescence emission upon addition of DNA. Also, the relatively high number of free poly-Lysine sites available allows binding of DNA without displacement of the porphyrins (no increase in the fluorescence intensity), thus introducing negative errors in the assay,

especially in the low DNA concentration range. For H_2TPPS concentrations > 10^{-6} M high amounts of free porphyrins are present in solution, leading to a high fluorescent background and reducing the sensitivity of the assay.

A second critical factor to be optimized is the ratio of poly-Lysine/H_2TPPS concentrations. A low ratio causes (i) a poor initial fluorescence quenching (high background signal), because only a small percentage of porphyrin can be obtained in the aggregated form and (ii) a low steepness, because a lower amount of porphyrin becomes displaced after each addition of DNA and, therefore, a low sensitivity of the method. On the other hand, high ratios of poly-Lysine/H_2TPPS permit to start out with extremely low values of initial fluorescence intensity (strong quenching). However, as discussed earlier, the system shows an 'inertia' not responding readily when the first few DNA aliquots are added until all binding sites on the polymer are saturated. From this point on the DNA starts displacing porphyrin molecules (which regain their fluorescence) and the system starts responding to further additions of DNA. Here, again, this effect causes a large negative error in the quantitative DNA determination, especially in the low DNA concentration range which would be interesting for analytical applications. Empirically it was determined that best poly-Lysine/H_2TPPS ratios ranged between 10 and 15.

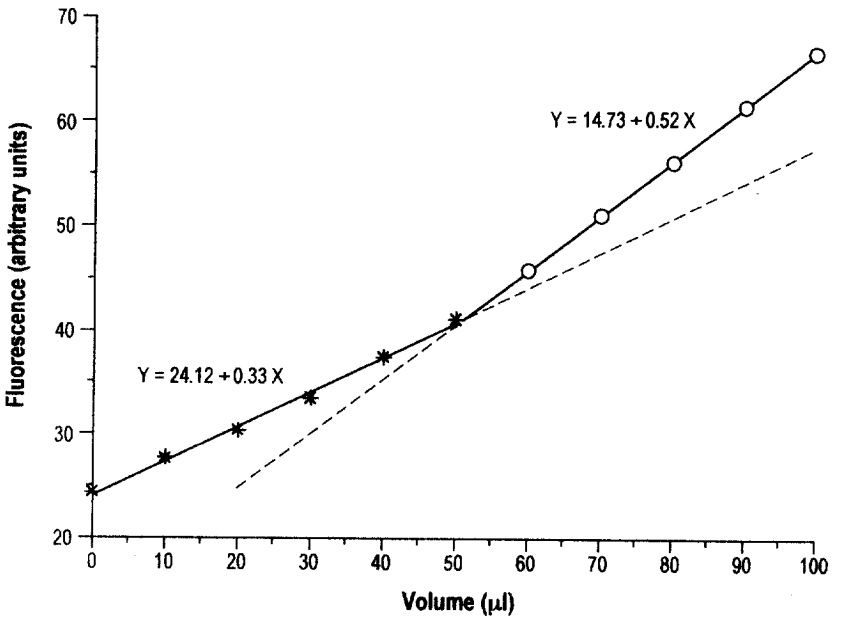

Fig. 12. Variation of the fluorescence intensity of the H_2TPPS/poly-Lysine system upon addition of successive aliquots of a DNA solution of known concentration followed by the addition of the same aliquots of a DNA sample of unknown concentration. $H_2TPPS = 1 \times 10^{-6}$ M. Poly-Lysine = 1.3×10^{-5} M. $\lambda_{exc} = 414$ nm. $\lambda_{em} = 640$ nm. Reprinted from Ref. [40] with kind permission of Elsevier Science.

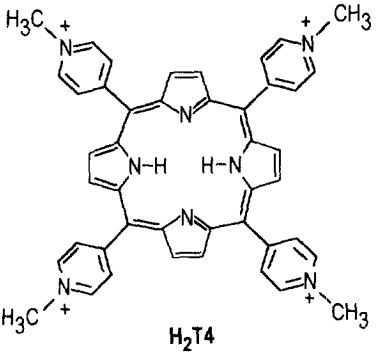

Fig. 13. Molecular structure of the *meso*-tetrakis(4-*N*-methylpyridyl)-porphine (H_2T4).

A third important factor to be optimized is the ionic strength (μ) because both H_2TPPS and DNA binding to poly-Lysine is mostly driven by electrostatic interactions. In fact, for high ionic strengths the system is characterized by a high fluorescence background while for low values of μ the interaction of H_2TPPS with the polymer chain is so strong that becomes difficult for the DNA to displace the porphyrin from the matrix. A good compromise which gives best results is reached for ionic strengths in the range of 30–50 mM.

4. Determination of nanomolar concentrations of zinc(II) and copper(II) using supramolecular complexes of H_2T4 on poly-Glutamate

Finally, a selective and very sensitive supramolecular sensor for biologically relevant metal ions such as zinc(II) and copper(II) has been obtained by means of extremely simple routes [41]. The system employed for such application is a supramolecular complex formed between the tetracationic *meso*-tetrakis(4-*N*-methylpyridyl)-porphine (H_2T4) (Fig. 13) and the polyanionic poly-Glutamate. In this particular case, the spectroscopic variations following porphyrin metallation were used as analytical tool. In fact, in most cases, the absorption and/or the emission properties of the metallo- and the free-porphyrins are quite different from each other, so that the two species can be clearly distinguished. For example, absorption and emission maxima for ZnT4 and H_2T4 are $\lambda_{exc} = 436$ nm, $\lambda_{em} = 622$ nm, and $\lambda_{exc} = 422$ nm, $\lambda_{em} = 655$ nm, respectively. On the other hand, the insertion of copper(II) causes a complete quenching of the fluorescence of H_2T4 (a weak emission, centered at about 770 nm, is observed in water, but only when CuT4 is intercalated into natural or synthetic DNAs) [42]. In spite of that, metallation reactions have been rarely used for analytical applications [43–46], a major limitation being the slow rate of metal insertion. In contrast, we have observed, for the supramolecular complex formed by H_2T4 and poly-Glutamate, a remarkable increase of porphyrin metallation reaction rate, allowing this complex to behave as sensitive and specific fluorescent sensor for the determination of nanomolar concentrations of copper(II) and zinc(II).

Actually, we should underline that, since Zn and Cu porphyrin metallation are non reversible processes (and therefore do not allow continuous monitoring), we should more properly refer to these systems as dosimeters or indicators but the word 'sensor' is employed for simplicity.

Under the experimental conditions used, H_2T4 is mainly monodispersed on the anionic polypeptide. In fact, only a small red shift ($\Delta\lambda = 4$ nm) and hypochromicity ($\approx 15\%$) of the Soret band is observed, while any characteristic feature of porphyrin assembly formation (such as Resonance Light Scattering or induced circular dichroism signals in the Soret region) is absent [47]. Absorption experiments show that, the addition of zinc(II) to such H_2T4-poly-Glutamate supramolecular complex, leads to a shift of the Soret band at 440 nm (Fig. 14). Also, the main Q-band of H_2T4 (518 nm) is progressively replaced with that of ZnT4 (562 nm) (Fig. 14). These spectroscopic features are diagnostic of the formation of ZnT4. Noteworthy, the addition of successive aliquots, each containing few picomoles of zinc(II), leads to a linear increase of the fluorescence intensity at 622 nm (ZnT4 emission maximum), as shown in Figs. 15 and 16.

The presence of poly-Glutamate turns out to be essential to observe a reasonable time response of the method. In the presence of the anionic polypeptide, zinc(II) insertion is completed in about 10 min whereas in its absence, even after 24 h no evidence of metallation can be observed; i.e. the formation of the poly-Glutamate-H_2T4 supramolecular complex 'catalyzes' the insertion of zinc(II).

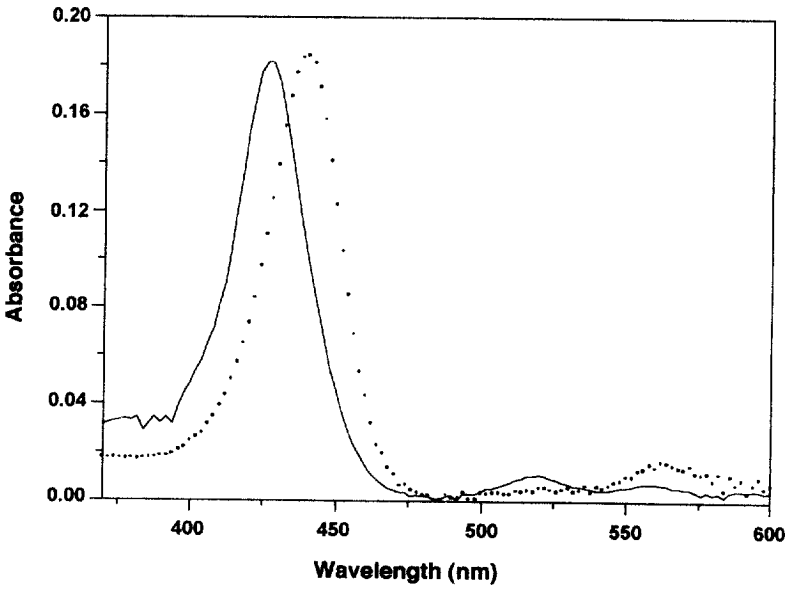

Fig. 14. Absorption spectra of H_2T4 (1 μM) with poly-Glutamate (200 μM) in succinate buffer (2 mM) at pH 5.6; before (—) and after (...) the addition of $ZnSO_4$ (1 μM). Reprinted from Ref. [41] with kind permission of the Royal Society of Chemistry.

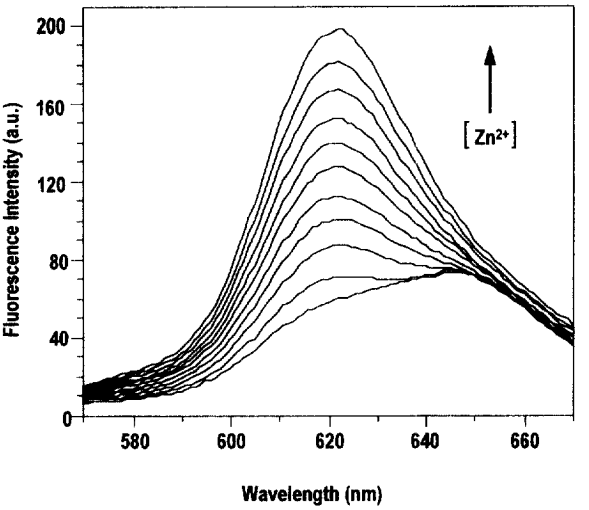

Fig. 15. Fluorescence emission spectra for a solution of H_2T4 (1 μM) with poly-Glutamate (200 μM) in succinate buffer (2 mM) at pH 5.6 and for increasing concentrations of zinc(II) in the range 2×10^{-9} to 1×10^{-7} M. In order to minimize the contribution of H_2T4 fluorescence, the excitation wavelength used in these experiments is 445 nm. Modified from Ref. [41] with kind permission of the Royal Society of Chemistry.

A similar behavior is observed for the same system when Cu(II) is employed in place of Zn(II). Here, the formation of the metallo-porphyrin is confirmed by the gradual replacement of H_2T4 Q-band (518 nm) with that of CuT4 (550 nm), as Cu(II) concentration is increased. However, since the CuT4 formed is non-fluorescent, it is possible to monitor the disappearance of the fluorescence emission of H_2T4 mono-dispersed on poly-Glutamate. Here, as well, a plot of H_2T4 emission fluorescence intensity ($\lambda_{exc} = 422$ nm, $\lambda_{em} = 655$ nm) versus Cu(II) concentration (Fig. 17) shows a linear behavior.

Interestingly, also the pH dependence of the metallation rate is influenced by the presence of poly-Glutamate. In fact, it has been previously reported that, in the presence of nitrate, H_2T4 metallation rate is almost unaffected in the pH range 4.4–5.5 and accelerated at higher pHs [48,49]. On the contrary, in the presence of polyglutamate the metallation rate increases with pH (i.e. with the number of negative charges on the polypeptide) in the range ≈ 4.5–5.5 and then does not increase anymore at higher pHs. Also, at pHs lower than ≈ 4.5 the insertion reaction is not catalyzed by poly-Glutamate. These observations suggest that, unlike other simple ligands (such as acetate, pyridine, ammonia, nitrate), the 'catalytic' role of the anionic template must be related to its electrostatic field by (i) increasing the 'local' concentration of metal ions [50], and (ii) partially shielding the positive charges of the H_2T4 monodispersed on it (thus facilitating the approach of the cationic metal ions to the cationic porphyrins). Most likely, also the state of the porphyrins plays a role in the reaction rate trend observed at pHs lower than ≈ 4.5. H_2T4 is, in fact, aggregated on poly-Glutamate in the pH range 3.4–4.5, and is

monodispersed on the polypeptide both at higher and lower pHs [47]. In particular, aggregation should reduce the accessibility of the metal ions to the porphine 'core', owing to the porphyrins face-to-face arrangement [12,23]. This hypotheses is in good agreement with previous experiments by Pasternack et al. [51] which show that aggregation and/or intercalation of porphyrins onto or into synthetic DNA drastically reduces porphyrin metallation rate. These observations underline that both the nature of the matrix and of the supramolecular complex (i.e. of the molecular recognition processes leading to the supramolecular species) are crucial to allow the catalysis of the metal insertion by an anionic template.

Finally, the addition of other metal ions, such as Co(II), Fe(II), Mg(II), Mn(II), and Ni(II) does not cause any of the spectroscopic variations described above in a time interval comparable to that observed for Zn(II) and Cu(II). A reasonable explanation for this behavior is that the 'uncatalyzed' rate of insertion for as Co(II), Fe(II), Mg(II), Mn(II), and Ni(II) in H_2T4 is much lower than that of Zn(II) and Cu(II).

5. Concluding remarks and future developments

We have shown that the interactions of anionic porphyrins (such as H_2TPPS) with polymeric cationic matrices (such as poly-Lysine) can be modulated, both in terms of type and extent, by the charge density of the polymer chain and the steric

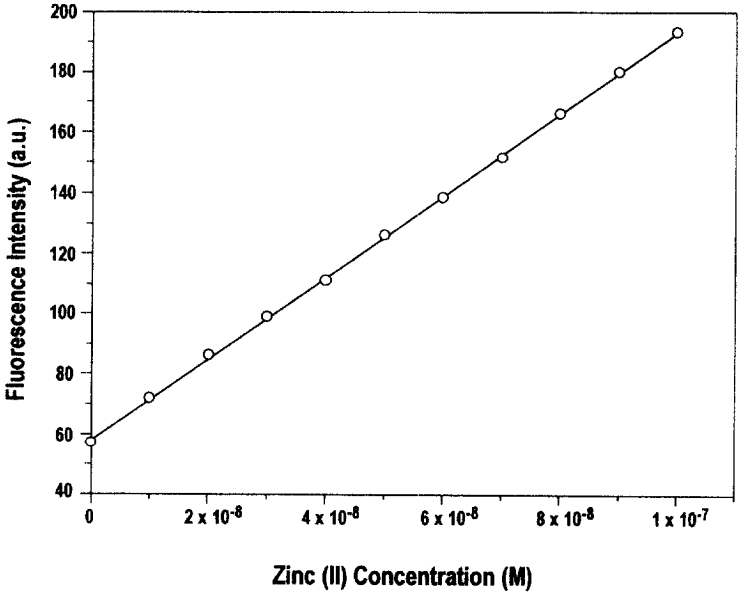

Fig. 16. Plot of fluorescence intensity at 622 nm ($\lambda_{ex} = 445$ nm) vs. zinc(II) concentration, for a solution of H_2T4 (1 μM) with poly-Glutamate (200 μM) in succinate buffer (2 mM) at pH 5.6. Modified from Ref. [41] with kind permission of the Royal Society of Chemistry.

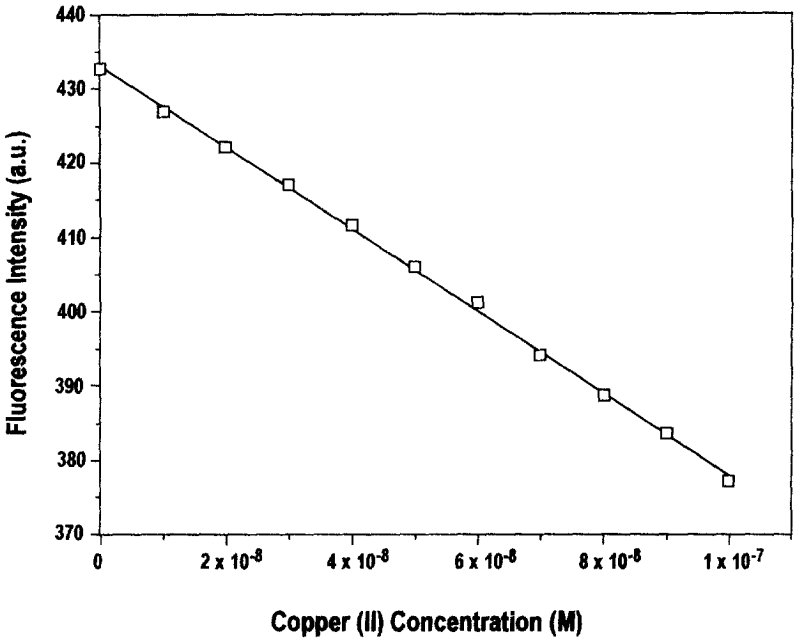

Fig. 17. Plot of fluorescence intensity at 655 nm ($\lambda_{exc} = 422$ nm) vs. copper(II) concentration, for a solution of H_2T4 (1 μM) with poly-Glutamate (200 mM) in succinate buffer (2 mM) at pH 5.6. Modified from Ref. [41] with kind permission of the Royal Society of Chemistry.

features of the porphyrins. The absence of severe steric constrains leads to assemblies which can behave as fluorescent pH-sensors. The stability towards pH of these supramolecular aggregates seems to be related to their size, which can be tuned by changing: (i) the poly-Lysine to porphyrin ratio; (ii) the poly-Lysine length or (iii) the steric features of porphyrins (e.g. the central metal ion), which mainly affect the lateral distribution of the porphyrins along the polymer matrix and hence the quenching. In order to understand the role played by weak non-covalent interactions and desolvation processes in stabilizing these complexes it will be necessary to investigate on the thermodynamics of such porphyrin self-assembly processes [52].

Also, employing the same H_2TPPS/poly-Lysine system we have developed a simple, rapid and accurate method for the quantitative determination of DNA in solution. The effect of the presence of material which could possibly interfere with the assay, such RNA, proteins, dilute detergents and protein denaturants still needs to be investigated. Such molecules are, in fact, commonly present in 'real' DNA samples.

Finally, a very sensitive and selective fluorescent sensor for metal ions was developed. The system employed is a very simple supramolecular complex formed between *meso*-tetrakis(4-*N*-methylpyridyl)-porphine (H_2T4) and poly-Glutamate. Here, as a result of a remarkable increase of the porphyrin metallation reaction

rate, it is possible to detect and quantitate biologically relevant metal ions such as zinc(II) and copper(II) present in nanomolar concentrations.

To date, the non-covalent nature of these species, and therefore their relatively low robustness, has hindered their application for the development of chemical devices. However, the results obtained are very promising and provide an excellent example of the wide field of potential applications of porphyrin assemblies on polymeric matrices owing to their flexible and tunable physico-chemical properties.

6. Experimental section

6.1. Materials

The porphyrins were purchased from Midcentury, *meso*-tetrakis(4-sulfonatophenyl)porphine (H_2TPPS) as sodium salt and *meso*-tetrakis(4-*N*-methylpyridyl)porphine (H_2T4) as chloride salt. Porphyrin metallation was performed by literature methods [53]. H_2TPPS, Sn^{IV}TPPS and ZnTPPS concentrations were determined using $\varepsilon_{414} = 5.33 \times 10^5$ M^{-1} cm^{-1}, $\varepsilon_{418} = 6.2 \times 10^5$ M^{-1} cm^{-1}, and $\varepsilon_{421} = 6.8 \times 10^5$ M^{-1} cm^{-1}, respectively. H_2T4 concentration was determined using $\varepsilon_{423} = 2{,}26 \times 10^5$ M^{-1} cm^{-1}. All solutions were prepared using water bidistilled on $KMnO_4$ and porphyrin stock and working solutions were stored at room temperature in the dark to avoid undesired photochemical reactions.

Poly-Lysine, poly-Glutamate of different lengths and *Calf thymus* DNA were obtained from Sigma. Poly-Lysine and poly-Glutamate were always prepared fresh by dissolving small amounts of, respectively, the sodium salt and the bromohydrate salt in water; their concentration was determined using $\varepsilon_{205} = 3300$ M^{-1} cm^{-1} for poly-Lysine and $\varepsilon_{205} = 3500$ M^{-1} cm^{-1} for poly-Glutamate in doubly distilled water [54] and expressed as moles of lysine or glutamate residues per liter.

Calf thymus DNA was stored in 1 mM phosphate buffer, pH 7, containing 10 mM NaCl. The concentration of DNA stock solution was obtained spectrophotometrically using $\varepsilon = 1.32 \times 10^4$ M^{-1} cm^{-1} at 260 nm and expressed in moles of base pairs/liter. All DNA quantitation experiments using the H_2TPPS/poly-Lysine system were carried out at room temperature employing as buffer Piperazine-*N*,*N*′-bis(2-ethansulphonic acid) disodium salt (PIPES) 5 mM, pH 6.1.

6.2. Spectroscopic measurements

UV-vis measurements were performed on a Hewlett-Packard HP 8452 spectrophotometer using 1 cm quartz cuvettes. Fluorescence experiments were carried out on a Jasco FP-777 spectrofluorimeter with the detector oriented at 90° relative to the light source and using 1 cm disposable methacrylate cuvettes to minimize poly-Lysine adsorption on their surface. For Resonance Light Scattering (RLS) measurements, 1 cm polystyrene cuvettes were employed on a SPEX F111 spectrofluorimeter with both incident and detected wavelengths scanned synchronously.

Acknowledgements

This work was partially supported by the 'Consiglio Nazionale delle Ricerche' (CNR), and the 'Ministero dell'Universita e della Ricerca Scientifica e Tecnologica' (MURST).

References

[1] A.P. de Silva, H.Q.N. Gunaratne, T. Gunnlaugsson, A.J.M. Huxley, C.P. McCoy, J.T. Rademacher, T.E. Rice, Chem. Rev. 97 (1997) 1515.
[2] L. Fabbrizzi, M. Licchelli, P. Pallavicini, A. Perotti, A. Taglietti, D. Sacchi, Chem. Eur. J. 2 (1996) 75.
[3] R.A. Bissell, A.P. de Silva, H.Q.N. Gunaratne, P.M.L. Lynch, G.E. M. Maguire, K.R.A.S. Sandanayake, Chem. Soc. Rev. 21 (1992) 187.
[4] R. Grigg, W.D.J.A. Norbert, J. Chem. Soc. Chem. Commun. (1992) 1298.
[5] R. Grigg, W.D.J.A. Norbert, J. Chem. Soc. Chem. Commun. (1992) 1300.
[6] J.-M. Lehn, in: H.-J. Schneider, H. Dürr (Eds.), Frontiers in Supramolecular Organic Chemistry and Photochemistry, VCH, Weinheim, 1991, pp. 1–28.
[7] R.F. Pasternack, E.J. Gibbs, in: A. Sigel, H. Sigel (Eds.), Metal Ions in Biological Systems, vol. 33, Marcel Dekker, Basel, 1996, pp. 367–397.
[8] S. Ikeda, T. Nezu, G. Ebert, Biopolymers 31 (1991) 1257.
[9] T. Nezu, S. Ikeda, Bull. Chem. Soc. Jpn. 66 (1993) 25.
[10] R. Purrello, S. Gurrieri, E. Bellacchio, R. Lauceri, L. Monsù Scolaro, in: P. Carmona, R. Navarro, A. Hernanz (Eds.), Spectroscopy of Biological Molecules: Modern Trends, Kluwer, Dordrecht, 1997, pp. 91–92.
[11] R. Purrello, E. Bellacchio, S. Gurrieri, R. Lauceri, A. Raudino, L. Monsù Scolaro, A.M. Santoro, J. Phys. Chem. B 102 (1998) 8852.
[12] C.A. Hunter, J.K.M. Sanders, J. Am. Chem. Soc. 112 (1990) 5525.
[13] C.A. Hunter, Angew. Chem. Int. Ed. Eng. 32 (1993) 1584.
[14] H.-J. Schneider, M. Wang, J. Org. Chem. 59 (1994) 7464.
[15] L.F. Newcomb, S.H. Gellman, J. Am. Chem. Soc. 116 (1994) 4993.
[16] E. Ojadi, R. Selzer, H. Linschitz, J. Am. Chem. Soc. 107 (1985) 7783.
[17] U. Hofstra, R.B.M. Koehorst, T.J. Schaafsma, Chem. Phys. Lett. 130 (1986) 555.
[18] E.B. Fleischer, J.M. Palmer, T.S. Srivastava, A. Chatterjee, J. Am. Chem. Soc. 93 (1971) 3162.
[19] K. Kalyanasundaram, M. Neumann-Spallart, J. Phys. Chem. 86 (1982) 5163.
[20] For example, the external binding of tetra-cationic porphyrins onto natural or synthetic DNA leads to small variations of the absorption spectra. On the other hand, porphyrin intercalation (not possible for our system) or aggregation induces more substantial spectral variations (see for example R.F. Pasternack, E.J. Gibbs, ACS Symp. Ser. 402 (1989) 59).
[21] E.J. Gibbs, I. Tinoco, M. Maestre, P. Ellinas, R.F. Pasternack, Biochem. Biophys. Res. Commun. 157 (1988) 350.
[22] G.A. Schick, I.C. Schreiman, R.W. Wagner, J.S. Lindsey, D.F. Bocian, J. Am. Chem. Soc. 111 (1989) 1344.
[23] J.-F. Fuhrhop, C. Demoulin, C. Boettcher, J. Köning, U. Siggel, J. Am. Chem. Soc. 114 (1992) 4159.
[24] R.F. Pasternack, P.J. Collings, Science 269 (1995) 935.
[25] M. Kasha, H.R. Rawls, M.A. El-Bayoumi, Pure Appl. Chem. 11 (1965) 371.
[26] A. Osuka, K. Maruyama, J. Am. Chem. Soc. 110 (1988) 4454.
[27] J.L. Hoard, Porphyrins and Metalloporphyrins, Elsevier, Amsterdam, 1975.
[28] T. Maniatis, E.F. Fritsch, J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1989.

[29] M.D. Dutton, R.J. Varhol, D.G. Dixon, Anal. Biochem. 230 (1995) 353.
[30] C.F. Brunk, K.C. Jones, T.W. James, Anal. Biochem. 92 (1979) 497.
[31] C. Labarca, K. Paigen, Anal. Biochem. 102 (1980) 344.
[32] C. Cesarone, C. Bolognesi, L. Santi, Anal. Biochem. 100 (1979) 188.
[33] R. Rago, J. Mitchen, G. Wilding, Anal. Biochem. 191 (1990) 31.
[34] H.S. Rye, J.M. Dabora, M.A. Quesada, Anal. Biochem. 208 (1993) 144.
[35] D. Marie, D. Vaulot, F. Partensky, Appl. Environ. Microbiol. 62 (1996) 1649.
[36] S.J. Ahn, J. Costa, J.R. Emanuel, Nucleic Acids Res. 24 (1996) 2623.
[37] V.L. Singer, L.J. Jones, R.P. Haugland, Anal. Biochem. 249 (1997) 228.
[38] C.Z. Huang, K.A. Li, S.Y. Tong, Anal. Chem. 68 (1996) 2259.
[39] M. Roza-Fernàndez, M.J. Valencia-Gonzàlez, M.E. Dìaz-Garcìa, Anal. Chem 69 (1997) 2406.
[40] S. Gurrieri, A. Aliffi, E. Bellacchio, R. Lauceri, R. Purrello, Inorg. Chim. Acta 286 (1999) 121.
[41] E. Bellacchio, S. Gurrieri, R. Lauceri, A. Magrì, L. Monsù Scolaro, R. Purrello, A. Romeo, J.Chem. Soc. Chem. Commun. (1998) 1333.
[42] B.P. Hudson, J. Sou, D.J. Berg, D.R. McMillin, J. Am. Chem. Soc. 114 (1992) 8997.
[43] C.V. Banks, R.E. Bisque, Anal. Chem. 29 (1957) 526.
[44] J.-I. Itoh, T. Yotsuyanagi, K. Aomura, Anal. Chim. Acta 74 (1975) 53.
[45] S. Funashi, Y. Ito, M. Inamo, Y. Hamada, M. Tanaka, Mikrochim. Acta I (1986) 33.
[46] M. Tabata, M. Kumamoto, J. Nishimoto, Anal. Chem. 68 (1996) 758.
[47] E. Bellacchio, S. Gurrieri, L. Monsù Scolaro, R. Purrello, to be submitted.
[48] P. Hambright, P.B. Chock, J. Am. Chem. Soc. 96 (1974) 3123.
[49] M. Tabata, M. Tanaka, Trends Anal. Chem. 10 (1991) 128.
[50] G.S. Manning, J. Phys. Chem. 85 (1981) 870.
[51] R.F. Pasternack, E.J. Gibbs, R. Santucci, S. Schertel, P. Ellinas, S.C. Mah, J. Chem. Soc. Chem. Commun. (1987) 1771.
[52] A. Raudino, S. Gurrieri, R. Lauceri, L. Monsù Scolaro, R. Purrello, in preparation.
[53] O. Hermann, S.H. Mehdi, A. Corsini, Can. J. Chem. 56 (1978) 1084.
[54] K. Rosenheck, P. Doty, Proc. Natl. Acad. Sci. USA 47 (1961) 1775.