FLUORESCENT LABELS GRAFTED ON POLY(ACRYLIC ACID) ACCOUNTING FOR CONFORMATIONAL CHANGES THROUGH NON-RADIATIVE ENERGY TRANSFER

Simona-Antoanela GĂVAN and Dan F. ANGHEL*

Department of Colloids, "Ilie Murgulescu" Institute of Physical Chemistry, Spl. Independenţei 202, 060021 Bucharest, Roumania

Naphthalene and pyrene separately grafted on poly(acrylic acid) (PAA) are used to account for conformational changes induced by pH through non-radiative energy transfer (NRET). When the separation of chromophores is within the range of NRET, excitation at 290 nm resulted in emissions from both naphthalene and pyrene excited by energy transfer, and proves the existence of polymeric interaction. A decrease in naphthalene emission and a concomitant increase of pyrene emission appear as the polymer solution is brought from low to high pH denoting polymer conformational changes. Complementary data from excitation and absorption spectra attest the presence of ground state excimers in labeled PAA. Although the polymer concentrations are far below from the overlapping value, the NRET results testify the entanglement of chains belonging to different macromolecules. The process is more intense in alkaline than in acidic medium, and is due to changes in polymer conformation.

INTRODUCTION

Non-radiative energy transfer (NRET) is a powerful technique for characterizing distance-dependent interactions on a molecular scale. It is one of the few tools able to measure inter- and intra-molecular interactions both in-vivo and in-vitro. NRET originates in dipole-dipole interactions between an energy donor in excited state and an energy acceptor in ground state. The technique is suitable to study events appearing over a distance that ranges from 1 to 10 nm, and is used to characterize polymers,[1-3] micelles,[4,5] nanoparticles,[6,7] membranes,[8,9] and biological systems.[10,12] It is also appropriate to develop biosensors,[11,12] and drug-screening protocols.[13-16]

Recently, a new class of water-soluble polymers, known as associating polymers (APs) or hydrophobically-modified polymers was introduced.[17] APs are water soluble polymers carrying a small number of long alkyl or perfluoroalkyl groups. They act as solution thickeners, and highly efficient gelation or viscosification agents in a variety of industrial fluids.[18] Although the macroscopic properties of APs are evident, the data gathered by rheometry,[19] surface tension,[20] and recently by a score of methods including viscometry, ^{1}H NMR, absorbance and birefringence[21] or surface tension, scanning electron microscopy, rheometry and dynamic light scattering[22] do not fully explain the phenomena occurring at molecular scale in these systems. To shed more light on APs solutions, fluorescently labeled polymers (FLPs) are proposed.[23] By monitoring changes in FLPs photophysical properties it is possible to assess the molecular basis of APs macroscopic behavior.
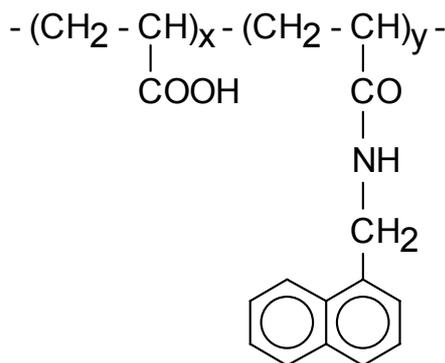
Previously we labeled the poly(acrylic acid) (PAA) with pyrene (Py), naphthalene (Np) or both fluorophores, and photochemically characterized it.[24,25] The effect of surfactants on these polymers was also investigated.[26,27] The main conclusion of those studies was that the labeled fluorophores form ground-state aggregates that depend on solvent. In water, the aggregates are affected by pH, ionic strength, and other external stimuli like surfactants added into system. In this work we examine the effect of pH on mixed aqueous solutions of PAA-Np and PAA-Py. These chromophores are known to interact as energy
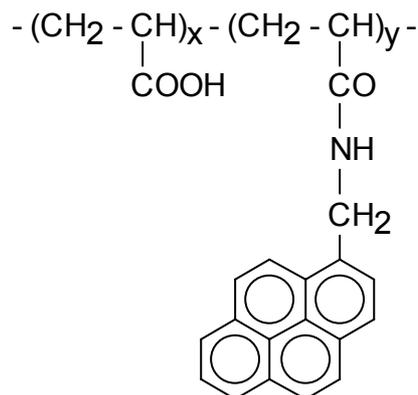
* Corresponding author: adan@icf.ro

donor (Np) and energy acceptor (Py). To further characterize these systems, fluorescence excitation and UV-Vis spectra were recorded.

EXPERIMENTAL

The synthesis and characterization of pyrene- and naphthalene-labeled PAA was done elsewere.[24,25] Fig. 1 shows the structures of labeled polymers. The figures assigned to

polymer acronyms denote the number of monomeric acrylic acid units per fluorophore.

The fluorescence measurements were done with a Fluoromax 4 (Horiba Jobin Yvon) fluorimeter. The absorbance spectra were recorded on a Cary 100 (Varian) spectrophotometer. The solutions were prepared with Millipore water (Simplicity UV water purification system). The pH was monitored with an ORION pH-meter, model 420A. The measurements were done at 25 °C.

- (CH$_2$ - CH)$_x$ - (CH$_2$ - CH)$_y$ -
  | |
  COOH   CO
                      |
                      NH
                      |
                      CH$_2$

PAA-Np/52

- (CH$_2$ - CH)$_x$ - (CH$_2$ - CH)$_y$ -
  | |
  COOH   CO
                      |
                      NH
                      |
                      CH$_2$

PAA-Py/32

Fig. 1 – The chemical structures and acronyms of the labeled polymers.

RESULTS AND DISCUSSION

Photophysical characterization of PAA-Np/52 and PAA-Py/32

Before discussing the effect of pH on mixed PAA-Np/52 and PAA-Py/32 aqueous solutions, we examined separately their photophysical properties. Fig. 2 illustrates the emission spectra of PAA-Np/52 at pH 3 and 11, normalized at the (0,0) transition. The spectra have both monomer and excimer emission. In acidic medium, the monomer emission appears in the 310–340 nm range. It has the (0,0) transition situated at 340 nm (intensity I_M). A small peak at 328 nm precedes the maximum, and a shoulder succeeds it within the 350–353 nm range. The excimer becomes visible from 360 nm to 450 nm. It is not structured and has the emission maximum at 399 nm (intensity I_E). At basic pH, the (0,0) transition of monomer is situated at 339 nm being preceded and succeeded by shoulders located at 326–329 nm and 349–352 nm, respectively. The excimer emission is also present, but it is severely diminished and does not show the maximum visible at pH 3. The presence

of excimer in the emission spectra of PAA-Np/52 attests that the grafted naphthyl groups form aggregates protected from the hostile aqueous medium by the hydrophilic PAA backbone. Since the excimer emission diminishes with pH and that of monomer increases reveals the unwinding of the PAA-Np/52 random coil as long as the polyacid neutralizes and acquires negative charges.

Fig. 3 shows the normalized emission spectra of PAA-Py/32 at pH 3 and 9. They have bands belonging to both monomer and excimer. At pH 3, the monomer emission (intensity I_M) is produced by locally isolated pyrenes and has the (0,0) transition or I_1 band situated at 374.5 nm. Although grafted to PAA, the pyrene monomer emission preserves the fine vibronic structure of monomer emission specific to pyrene probe.[28] This is because the fluorophore is linked to the polymer via a short spacer, the methylene group, which allows conjugation of the π electrons of pyrene with those of amide group. The I_1 band of PAA-Py/32 at pH 3 is red-shifted by 4.2 and respectively 3.5 nm in comparison with the I_1 band of pyrene probe in water, and in surfactant micelles.[29] The (0,0) transition is followed by two small peaks at

379.5 (I_2) and 385.5 nm (I_3), and by a strong peak at 395 nm (I_5). These peaks are in turn red-shifted by 6, 4.9 and 4.7 nm with respect to those of the probe in water (I_2 = 373.5 nm, I_3 = 380.6 nm and I_5 = 390.3 nm) However, the I_4 peak appearing at 387.2 nm in the monomer emission of probe is not present in the emission of PAA-Py/32, and we ascribed this loss of sensitivity to grafting. The

excimer emission (intensity I_E) appears as a large, unstructured band, with the maximum centered at 481 nm. At pH 9, the emission spectrum is similar to that at pH 3. However, there are two main differences: the monomer emission is blue-shifted by 1 nm and the excimer emission is considerably smaller.
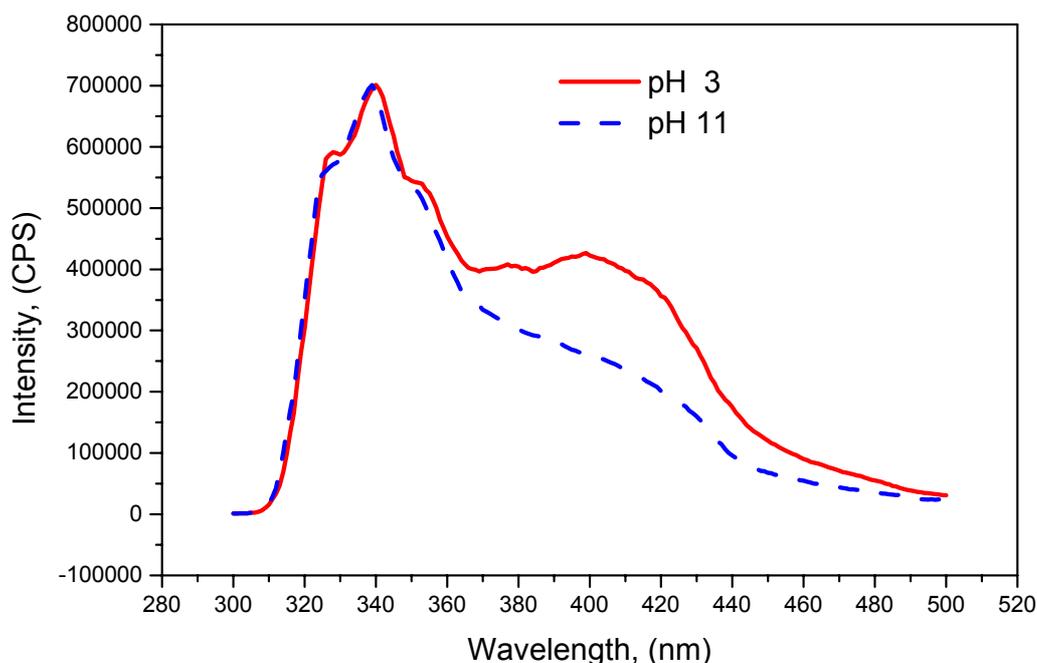

Fig. 2 – Normalized emission spectra of PAA-Np/52 (0.05 g/L). λ_{ex} = 290 nm.
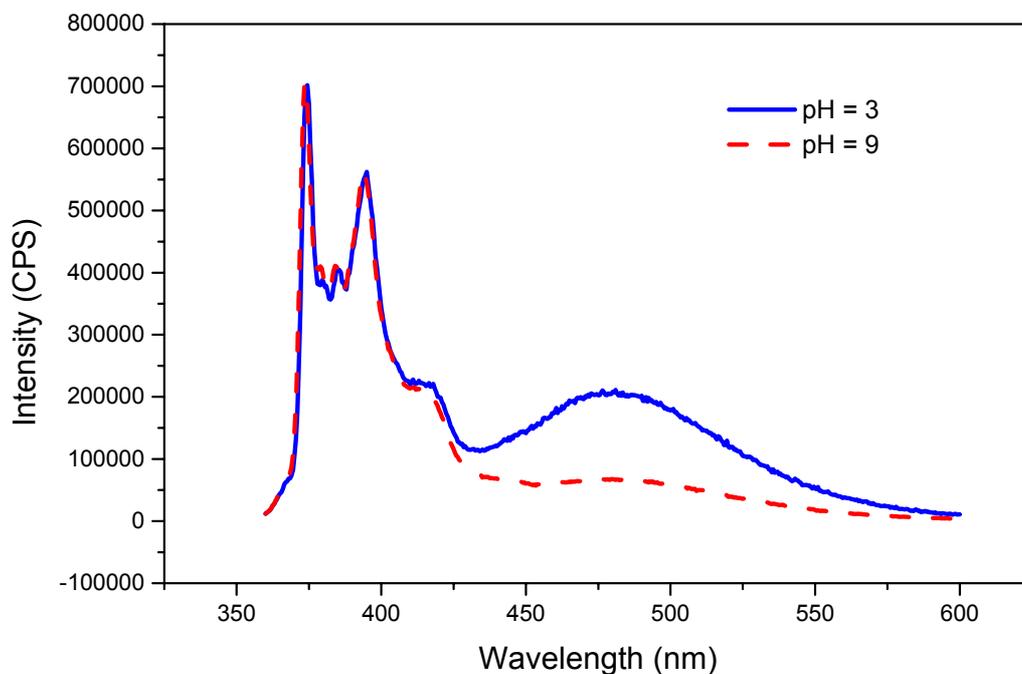

Fig. 3 – Normalized emission spectra of PAA-Py/32 (0.01 g/L). λ_{ex} = 344 nm.

The spectra in Figs. 2 and 3 show the decrease of excimer emission at alkaline pH. The decrease is accompanied by the increase of monomer emission, which is not evident in the Figs. 2 and 3 because the spectra are normalized to the (0,0) transition of monomer at pH 9. To explain these results, one has to admit that the conformation of labeled PAA in water depends on pH. At acidic pH, almost all monomeric units are not dissociated and the random coil is compact. It is more compact than the random coil of parent PAA because the pyrene labels bring together to protect from the hostile water.[24,25] Since the pyrene groups are close to each other, excimer emission is strong and arises mainly from intramolecular pyrene aggregates before excitation. At pH 9, the polymer is negatively charged, and its random coil unwinds by the repulsive electrostatic forces between the carboxylate groups. In this stretched conformation, there are less ground state aggregated pyrenes, denoted by the significant decrease of excimer emission. The low excimer emission mainly results from dynamic excimers formed by the encounter of an excited with a ground state pyrene attached to another macromolecule. In other words, the interactions taking place at high pH between the grafted pyrenes are preponderantly inter-macromolecular. However, there is still possible to exist ground-state and dynamic inter- and intra-macromolecular excimers.

Table 1

Parameters derived from fluorescence excitation and UV-Vis data at acidic and alkaline pH

	pH 3	pH 9
UV-Vis spectrum Excitation spectra	$P_A = 1.69$ $P_M = 2.25$ $P_E = 1.56$ $\Delta\lambda = 2.5$ nm	$P_A = 2.29$ $P_M = 2.21$ $P_E = 1.96$ $\Delta\lambda = 0.5$ nm

To document the above statements we recorded the absorption and excitation spectra. They give compelling information about pyrene preassociation. The electronic spectra (not shown) reveal a shift of the ^1L_a band toward lower wavelengths (hypsocromic effect) when pH increases, which denotes that pyrenes become more exposed to water. The peak-to-valley ratio for the (0,0) transition in ^1L_a band (P_A), equals 1.69 at pH 3 and 2.29 at pH 9 (see Table 1). Both are smaller than 3.0, the usual value for 1-subtituted pyrenyl compounds in absence of preassociation. The smaller P_A, the higher is pyrene preassociation.[30]

Excitation spectra give additional information concerning the ground-state interactions of labeled pyrenes. Although not shown, the spectra recorded at the monomer emission and at the excimer emission are clearly different. They have similar overall features, but do not superimpose. The spectrum monitored at the excimer emission is red-shifted compared to the spectrum monitored from the monomer, and the bands in the spectrum monitored for the excimer are broadened. The relative measures of these effects are the parameters presented in Table 1. At pH 3, the peak-to-valley ratio for the (0,0) transition in ^1L_a band in the excitation spectrum viewed at the monomer emission (P_M) is 2.25. The same parameter viewed at the excimer emission (P_E) is 1.56, and the difference in wavelength maxima for the (0,0) transition in ^1L_a band in the excitation spectra viewed at the monomer emission and at the excimer emission, $\Delta\lambda = 2.5$ nm. At pH 9, $P_M = 2.21$, $P_E = 1.96$, and $\Delta\lambda = 0.5$ nm. The increase of pH has little effect on P_M, but P_E raises and $\Delta\lambda$ decreases. When pyrenes do preassociate, P_M, is always higher than P_E, and $\Delta\lambda$ has positive values from 1 to 4 nm,[30,31] although larger shifts have been observed.[32] These results corroborated with those from electronic spectra support the idea that the aggregation degree of the pyrenes grafted on PAA is high at low pH and decreases when pH increases. Although the polymer becomes negatively charged at high pH, the electrostatic repulsion is not strong enough to overcome the hydrophobic forces keeping together the grafted pyrenes. The recorded P_A, P_M, P_E and $\Delta\lambda$ parameters attest that in the PAA-Py/32, the ground-state associated pyrenes decrease at alkaline pH, but they do not completely disappear. The statement is also valid for PAA-Np/52 as previously attested.[26]

NRET in mixed PAA-Np/32 and PAA-Py/52 systems

The idea that stems from the results presented above is that both PAA-Np/52 and PAA-Py/32 have pH sensitive conformation in aqueous solution. The random coils of the polymers are

shrunk at low pH, and adopt an open structure at high pH. In the latter case, although the experiments in the present study are conducted at very small polymer concentration, there is highly probable that naphthalene and pyrene grafted on separate PAA chains encounter to each other. According to this inference, there is little probability for Np to encounter Py when the grafted polymers are collapsed. To verify these assumptions we undertook NRET experiments at various pH values.

The prerequisite condition for NRET is the overlap of donor-emission and acceptor-absorption spectra. Other key parameters for NRET to occur are the distance separating the fluorophores and to a lesser extent their orientation. The perquisite condition was ascertained first and the results are presented in Fig. 4. It shows the emission spectrum of PAA-Np/52 and the UV-Vis spectrum of PAA-Py/32, at pH 3. The spectra superimpose very well, proving that the prerequisite NRET condition is fulfilled.
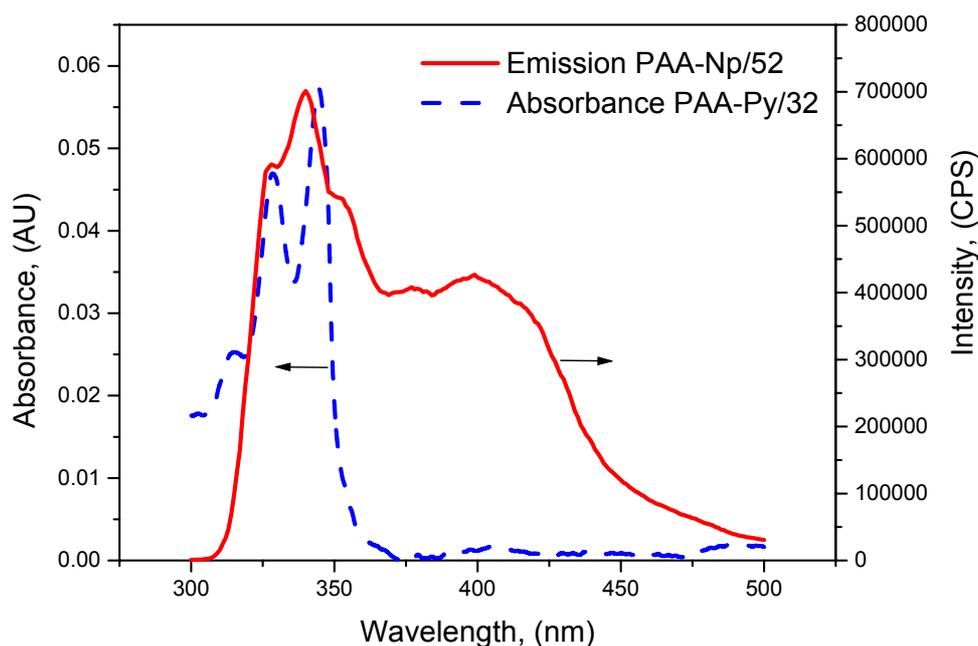

Fig. 4 – The absorption and emission spectra of labeled PAAs at pH 3.

Fig. 5 illustrates the emission spectra recorded during the NRET experiments conducted at pH 3 and 9. In both spectra two emissions appear. The first is in-between 310 and 400 nm and resulted from directly excited naphthalene (Np*). The second is due to Py* ($\lambda_{em} > 380$ nm) excited by energy transfer from Np*. These experiments prove that irrespective of pH, the distance separating the chromophores falls within the NRET range. When excitation was set at 346 nm (data not shown), it produced only the emission of pyrene, with no contribution from naphthalene.

A common measure of NRET is the ratio between the pyrene and naphthalene emission intensities (I_{Py}/I_{Np}). The I_{Py}/I_{Np} data obtained at various pH values are presented in Fig. 6, together with those of excimer-to-monomer emission ratio (I_E/I_M) for PAA-Py/32. Within the 2.5–4.8 pH range, the I_{Py}/I_{Np} is almost constant. Thereafter, it

increases steeply, and levels off above pH 7. In the same pH range, the I_E/I_M has an opposite trend. It is high and almost constant at low pH, starts decreasing around pH 4 and flattens above pH 7. The values associated with each data point of I_E/I_M represent the position of the (0,0) transition of ^1L_a band in the absorption spectrum. As stated in the previous chapter, it undergoes a hypsochromic shift with increasing pH, which indicates that pyrenes become progressively exposed to water by rising pH.

The obtained results can be rationalized in terms of conformational changes and interpolymeric association experienced by the labeled PAA when the pH changes (see Fig. 7). In this respect, one has to recall that both naphthalene and pyrene are hydrophobic compounds that do not like water. When grafted onto PAA, they try to avoid the contact with water by wrapping with the

hydrophilic polymer backbone. Therefore, both PAA-Np/52 and PAA-Py/32 have a shrunken conformation at low pH. By increasing pH, the carboxylic groups of PAA begin to dissociate and the polymer acquires negative charges. The random coil unfolds and takes a more and more opened conformation. As the polymer coil expands, chains labeled with different fluorophores have a higher probability to collide and NRET grows in intensity. Simultaneously, the excimer emission decreases because the population of ground-state associated pyrenes lowers.
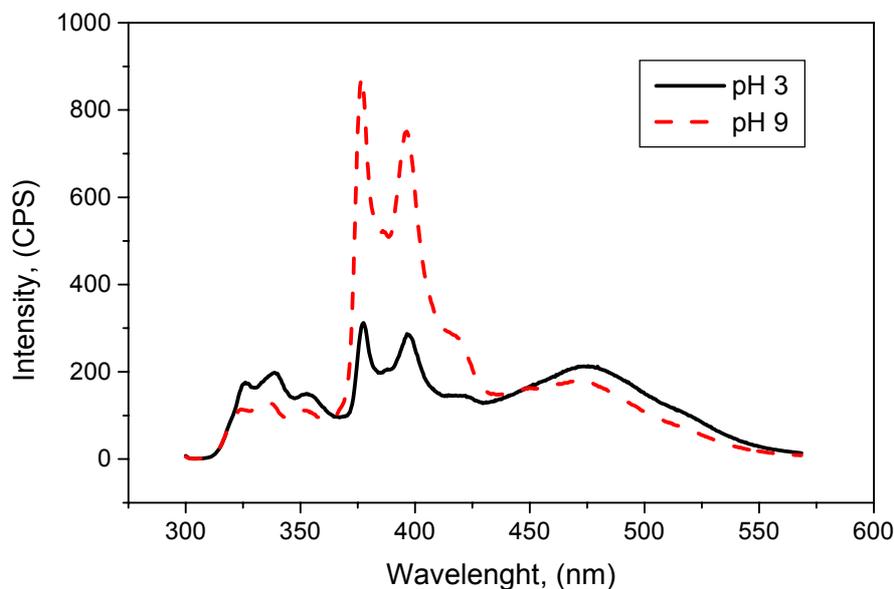

Fig. 5 – NRET at acidic and basic pH. Polymer concentration: PAA-Np/52 = 0.05 g/L, PAA-Py/32 = 0.01 g/L. λ_{ex} = 290 nm.

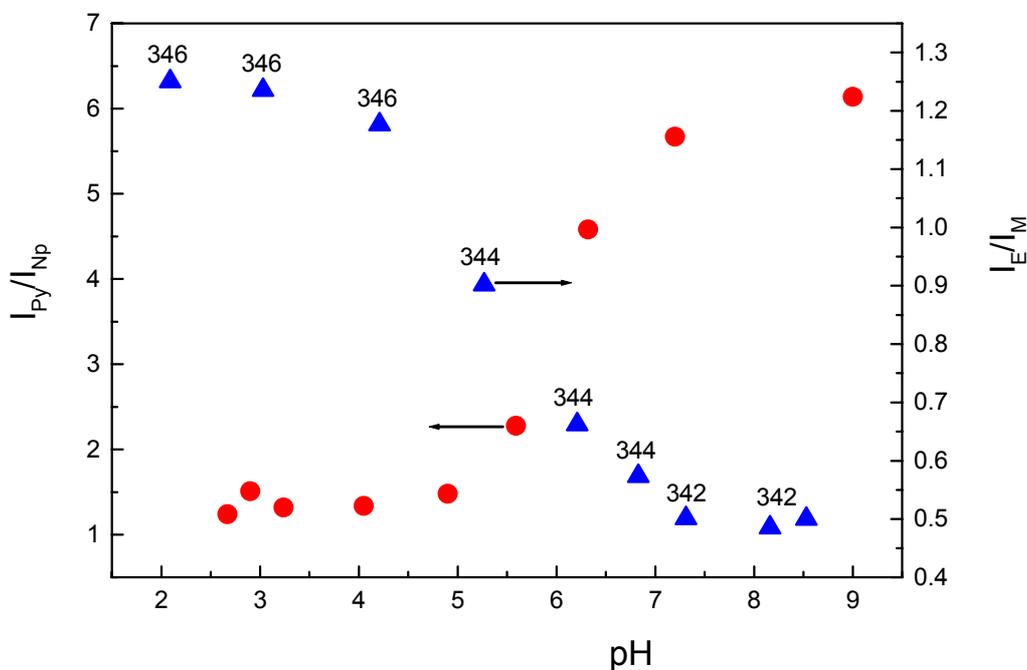Fig. 6 – Plots of NRET between PAA-Np/52 and PAA-Py/32 (λ_{ex} = 290 nm) and of I_E/I_M for PAA-Py/32 as a function of pH. The figures assigned to the points represent the position (in nm) of the (0,0) transition of the ^1L_a band in the UV absorption spectrum, the excitation being set at the respective value.
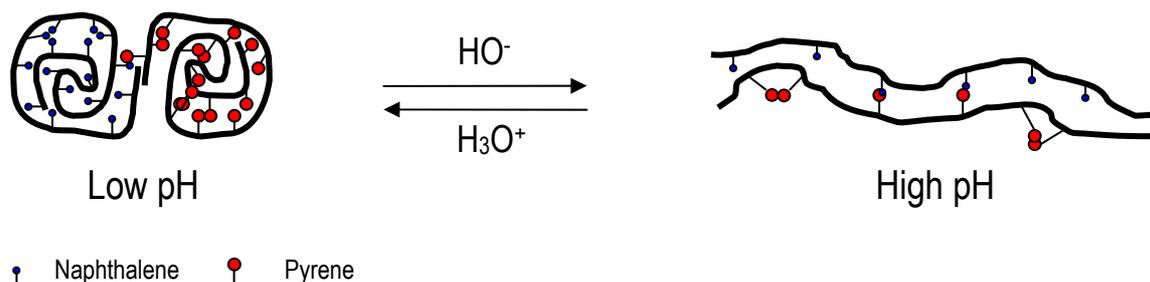
Fig. 7 – Sketches of the possible structures in PAA-Np/52 and PAA-Py/32 mixed aqueous solutions at acidic and basic pH according to NRET data.

CONCLUSIONS

The obtained results show that steady-state fluorescence is a suitable and powerful tool to study mixtures of fluorescently-labeled poly(acrylic acid)s. Although the polymer concentrations are far below the overlapping value, the obtained NRET results reveal profound pH induced structural changes. The changes in the photophysical properties of the grafted dyes can be traced on the basis of the well-known unwinding of the polyelectrolyte chains with increasing pH. At the same time, they lend support to understanding the properties of another important class of compounds, the associative polymers. They are nowadays largely used in many industrial and daily life fields. Therefore, a deeper insight and a better knowledge of properties of the associative polymers will improve the quality of the existing goods and will generate better new products.

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