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Dedicated to Professor Alexandru T. Balaban on the occasion of his 85th anniversary

SYNTHESIS AND FLUORESCENCE PROPERTIES OF SOME TRYPTOPHAN-CONTAINING POLYACRYLATES

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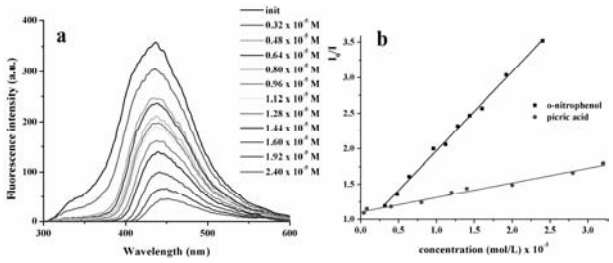
In this paper, two amino acid copolymers having fluorescent tryptophan in their structure were synthesized and characterized by means of Fourier-transform IR, proton nuclear magnetic resonance, UV-vis absorption and fluorescence spectroscopies. One of the copolymers, poly(AA-*co*-IA-*co*-ATrp) was prepared *via* free-radical polymerization of acrylic acid, itaconic acid and *N*-acryloyl-*L*-tryptophan in a molar ratio of 4:1:1 in the presence of 1,1'-azobis(cyclohexanecarbonitrile) as initiator. The second one was obtained by functionalization of the copolymer poly(AA-*co*-IA) with *L*-tryptophan methyl ester hydrochloride in the presence of *N,N*-carbonyldiimidazole as dehydrating agent. The fluorescent properties of the resulted copolymers were investigated using quenching tests with two nitro-aromatic compounds (2,4,6-trinitrophenol and *o*-nitrophenol). The experimental results suggested that these polymeric fluorophores could find applications for detection of some nitro-derivatives in *N,N*-dimethylformamide or 1,4-dioxane solution.



INTRODUCTION

Amino acid-based polymers have attracted significant research interest because of their ability to promote non-biological macromolecules with special properties which can be exploited in the development of novel biomaterials. Depending on the chemical nature of the amino acid moiety and its composition in the polymer structure, these copolymers display important flexibility for tuning of amphiphilicity, chirality, biological and self-assembly properties. Considerable recent progress in controlled/living radical polymerization techniques has allowed the synthesis of amino acid−based polymers with controlled molecular weights, narrow molar mass distribution, and complex architectures.[1-7] The use of α-amino acids may offer several advantages to the degradable polymers, such as improving the degradability and thermal, mechanical, and biological properties of polymeric materials. Additionally, the presence of chemical functionalities, such as hydroxyl, amine, carboxyl and thiol groups improves the hydrophilicity and allows possible interactions and

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modifications with other chemical species. Polymers of *N*-(meth)acryloyl-amino acids have been synthesized and studied by many research groups because of their relevance to proteins.[8,9] The typical synthesis of amino acid containing monomers uses (meth)acryloyl chloride in a coupling reaction with the desired amino acid to yield the corresponding (meth)acrylamides. By this synthetic route amino acid monomers with leucine, alanine, phenylalanine, glutamic acid, methionine, tyrosine, histidine, proline and tryptophan were prepared.[10-17]

L-Tryptophan, an essential amino acid and a vital constituent of proteins, has gained a great importance due to its ability to keep its fluorescence when coupled to other compounds and to lose it in the presence of quenchers. There have been published several reports on the synthesis of functional polymers with tryptophan moieties in their side chain. For example, Harada *et al.* reported on the interaction of a polymethacrylamide bearing hydrophobic tryptophan moieties, namely poly(*N*-methacryloyl tryptophan), with cyclodextrins.[9,18] The chiral recognition of methylated β-cyclodextrin by poly(*N*-isopropylacrylamide-co-(*D* or *L*)-*N*-tryptophan-acrylamide) was recently investigated.[19] Another research group dealt with the synthesis of tryptophan containing polymers followed by their modification with *L*-tryptophan methyl ester, and the study of its interaction with 1,1-bi-2-naphthol.[15] Methacrylate monomers having a chiral tryptophan moiety in the side chain, i.e., *Boc*-tryptophan methacryloyloxyethyl ester, afforded smart pH-responsive chiral cationic polymers.[16] Characteristic self-assembled structures such as micelles and inverse micelles, chiroptical properties and sensing functionality of the tryptophan-containing block copolymers were also investigated.[7]

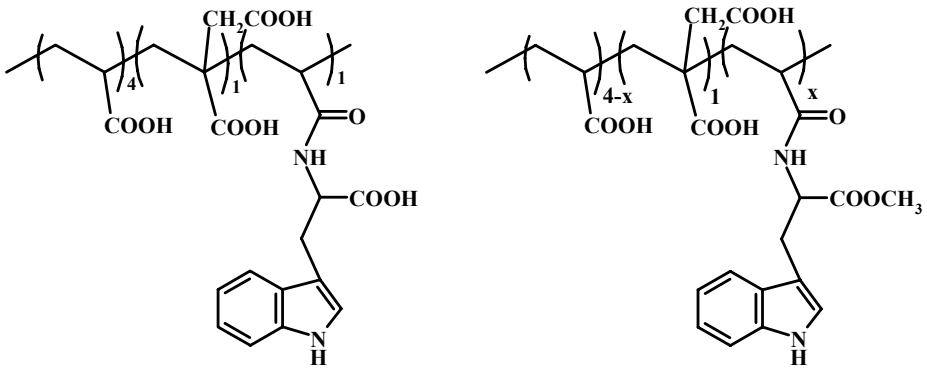
This contribution reports on the synthesis by conventional radical polymerization of novel tryptophan-based polymers, starting from acrylic acid, itaconic acid and *N*-acryloyl tryptophan, or acrylic acid and itaconic acid, further modified with tryptophan derivative, and their characterization including fluorescence emission in the presence of picric acid and *o*-nitrophenol as quenchers.

RESULTS AND DISCUSSION

Scheme 1 shows the chemical structure of the poly(AA-*co*-IA-*co*-ATrp) copolymer (PATrp), which is synthesized *via* the free-radical polymerization of acrylic acid, itaconic acid and *N*-acryloyl tryptophan in a molar ratio of 4:1:1 (AA:IA:ATrp). In the ^{1}H NMR spectrum of PATrp (Fig. 1) the signals positioned at higher field (1.48-2.33 ppm) correspond to the aliphatic protons of the macromolecular backbone, and the peak at 2.57 ppm is associated to the methylene protons from itaconic acid. The protons from indole ring are situated between 6.98 and 7.58 ppm, and those belonging to amide and amine group appeared in the zone of 8.30-8.42 ppm and 10.98-10.72 ppm, respectively. From the integral ratio of protons from tryptophan (a, b, c, d), methylene protons from itaconic acid and methylene/methyne protons from the backbone, the copolymer compositions was found to be 4:1:1, in good agreement with the feed ratio used in synthesis.

The FTIR spectrum of PATrp presented specific absorption bands that confirm the expected structure as follows: the characteristic bands for amide NH, carboxylic O-H, and aromatic and aliphatic CH stretching vibrations (3450-2600 cm^{-1}), carboxylic C=O stretching vibration (1730 cm^{-1}), amide C=O stretching vibration (1653 cm^{-1}), amide N-H stretching vibration (1541 cm^{-1}), amide C-N deformation vibration (1457 cm^{-1}), acyl C-O stretching vibration (1257 cm^{-1}) and methylene twisting band (C-H) (1115 cm^{-1}). Moreover, the absorption bands at 1651 and 811 cm^{-1}, which are ascribed to C=C bending vibrations of the starting monomers, were absent in the spectrum of the copolymer (Fig. 2).

It is well known that molecules with reactive amine groups can be attached to the polymer backbone possessing reactive carboxylic sites to form covalent conjugates *via* amide bonds in the presence of *N,N′*-dicyclohexylcarbodiimide (DCC). Based on this consideration the corresponding binary copolymer (poly(AA-*co*-IA)) was prepared and subsequently functionalized with *L*-tryptophan methyl ester hydrochloride (TrpME). The structure of this copolymer PATrpF (Scheme 1) was confirmed by its ^{1}H NMR spectrum, where additional signals of the methyl protons from ester were identified at 3.85 ppm. The functionalization degree was estimated from the integral ratio of tryptophan protons and the methylene/methyne protons from the polymer chain as being around 10%. Both copolymers are soluble in water, *N,N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO), tetrahydrofuran (THF) and *N,N*-dimethylacetamide (DMAc).



poly(AA-co-IA-co-ATrp) (PATrp) **poly(AA-co-IA) - TrpME (PATrpF)**

Scheme 1 – The structures of poly(AA-*co*-IA-*co*-ATrp) (PATrp) and poly(AA-*co*-IA)-TrpME (PATrpF).

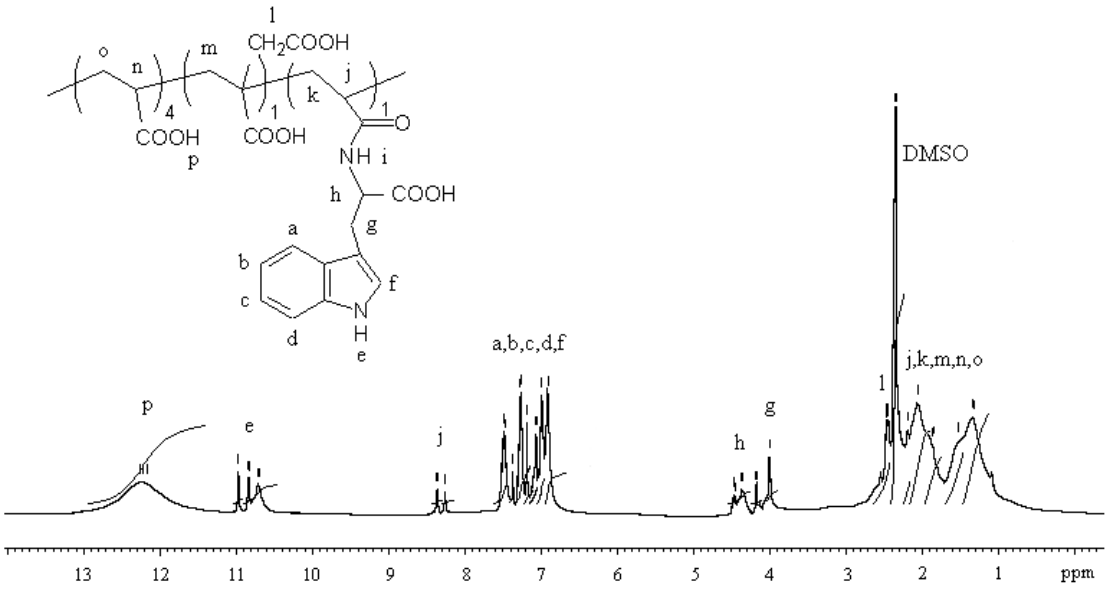


Fig. 1 – ^{1}H NMR spectrum of copolymer poly(AA-*co*-IA-*co*-ATrp) in DMSO-d_6.

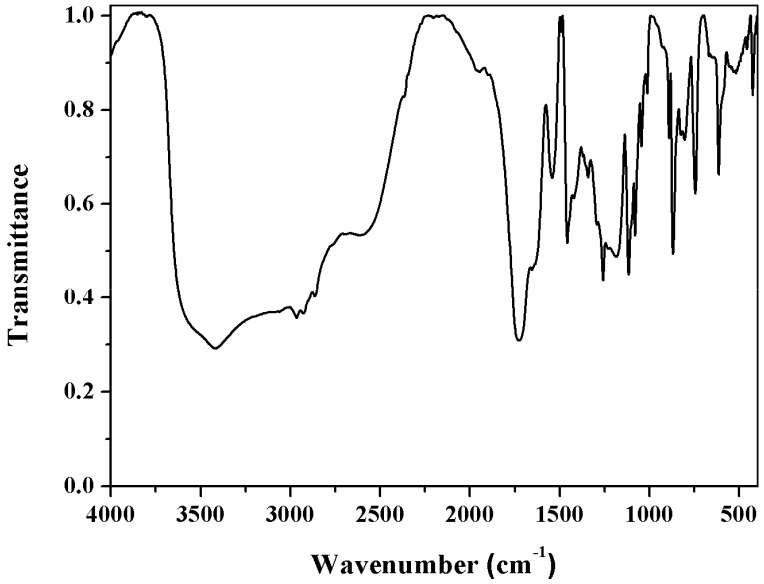


Fig. 2 – IR spectrum of poly(AA-*co*-IA-*co*-ATrp).

Since *L*-tryptophan is a fluorescent molecule, its presence on acrylic polymers confers them specific properties. In addition, hydrogen bonding between amide carbonyl oxygen and amide N-H hydrogen of the side chains and steric repulsion due to aromatic rings in these copolymers may also induce unique characteristics of *L*-tryptophan. Consequently, the UV-vis and fluorescence analysis of the obtained copolymers was carried out. UV-vis spectra of PATrp and PATrpF in DMF solutions exhibited similar shape and strong absorptions at 283 and 281 nm, respectively, attributed to the tryptophan chromophore. When these solutions were irradiated by the excitation wavelength at 280 nm, a strong emission band (~450 nm) and a shoulder (~350 nm) of the tryptophan moiety were recorded. The basic data revealed by fluorescence measurements gives information about the molecular environment around the chromophore. Therefore we evaluated the interaction of the above copolymers with various quenchers by monitoring the changes of the fluorescence intensity and the shift of maximum emission wavelength upon the addition of quenchers to the polymer solutions. The concentration of polymer solutions was of 2% in DMF or 1,4-dioxane, and picric acid and *o*-nitrophenol were used as quenchers. As can be observed in Fig. 3-5, the fluorescence intensity decreased remarkably as a result of the addition of quenchers to the polymer solutions and it was accompanied by a large shift of the maximum emission wavelength. For example, the emission maximum of PATrpF shifted from 450 to 485.5 nm at a concentration of 8.0×10^{-5} M of picric acid (Fig. 3) and from 446 to 486 nm by adding *o*-nitrophenol at a concentration of 2.4×10^{-5} M (Fig. 4). The both shifts of the emission maxima have close values, probably because of the similar structure of quenchers. Generally, this large displacement of the maximum emission wavelength is attributed to the hydrophilic or hydrophobic nature of the compounds. The electron deficient nitro-aromatic compounds (non- or weakly fluorescent products) are used as active compounds if a fluorophore unit is incorporated into their molecules, but especially as fluorescence quenchers acting in both dynamic and static processes.[20,21]

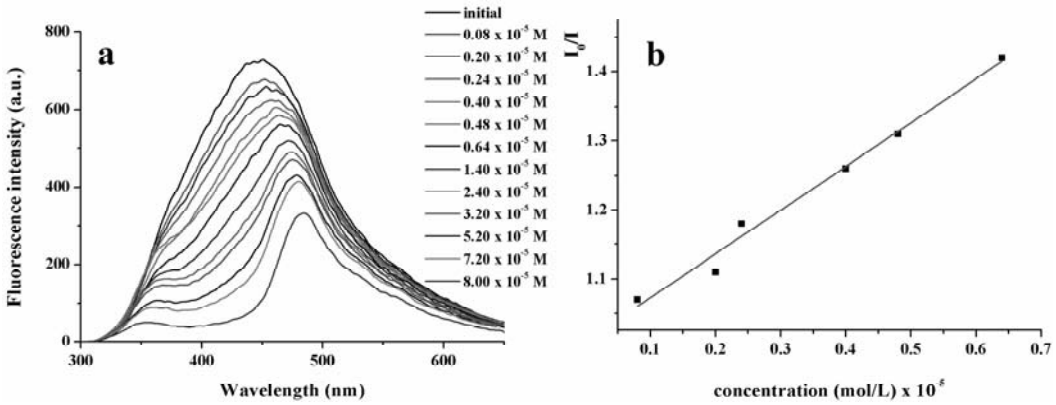


Fig. 3 – Fluorescence spectra of 2% copolymer PATrpF in DMF solution, in the absence and presence of picric acid at different concentrations (a); λ_{ex} = 280 nm; the Stern-Volmer plot (b).

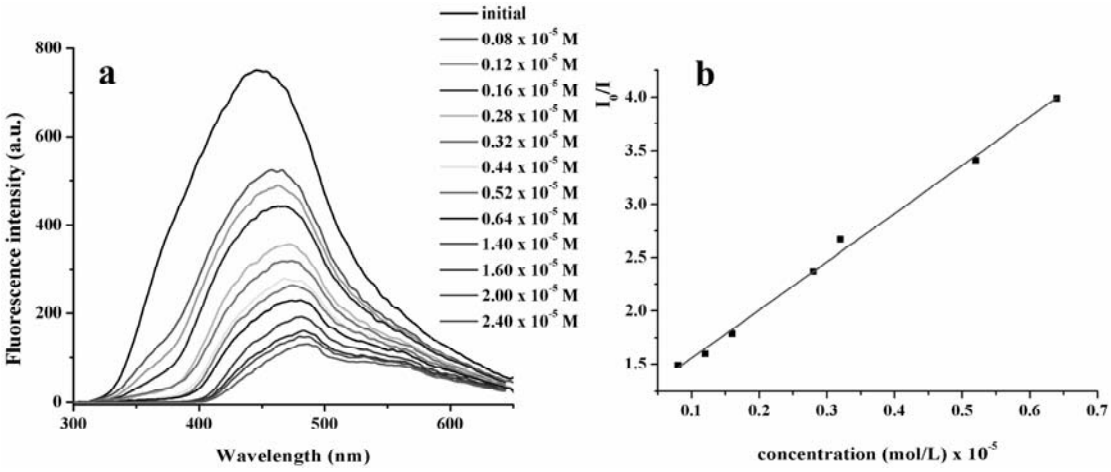


Fig. 4 – Fluorescence spectra of 2% polymer PATrpF in DMF solution, in the absence and presence of *o*-nitrophenol at different concentrations (a), λ_{ex} = 280 nm; the Stern-Volmer plot (b).

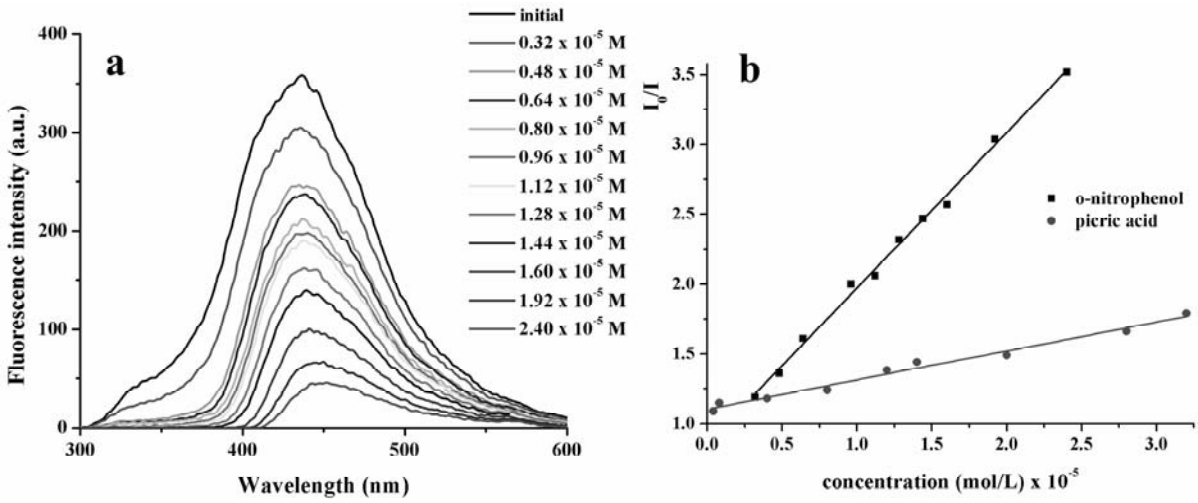


Fig. 5 – Fluorescence spectra of 2% polymer PATrp in dioxane solution, in the absence and presence of *o*-nitrophenol at different concentrations (a), λ_{ex} = 280 nm; the Stern-Volmer plots for fluorescence quenching with picric acid and *o*-nitrophenol (b).

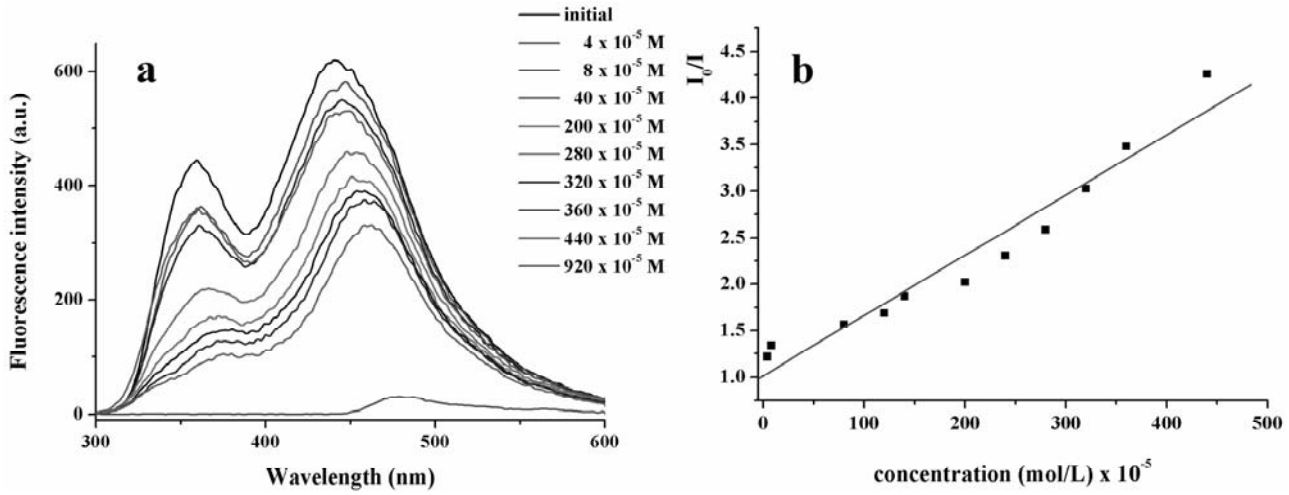


Fig. 6 – Fluorescence spectra of 1% polymer PATrp in dioxane solution in the absence and presence of picric acid at different concentrations (a), λ_{ex} = 280 nm; the Stern-Volmer plot (b).

Table 1

Fluorescence quenching characteristics obtained using linear regression Stern-Volmer Equation (1)

Polymer	Quencher	$K_{SV} = \frac{I_0}{I} - 1$ /Q (L/mol)	r^2	η (%)
PATrp	picric acid (dioxane)	0.20 x 10^5	0.982	68
PATrp	*o*-nitrophenol (dioxane)	1.11 x 10^5	0.995	87
PATrpF	picric acid (DMF)	0.30 x 10^5	0.995	54
PATrpF	*o*-nitrophenol (DMF)	3.20 x 10^5	0.931	83

By decreasing the polymer solution concentration at 1%, the emission spectra of PATrp in dioxane (Fig. 6) presented two maxima located at 359.5 and 450 nm, which were attributed to the monomer fluorescence and excimer fluorescence, respectively.[22]

Various mechanisms, such as proton transfer and electron transfer, long-range energy transfer, induced conformational changes and different intramolecular reactions such as excited state reactions, molecular rearrangements, ground-state complex formation or collision can explain the tryptophan fluorescence quenching by external quenchers. There are two quenching processes usually encountered, namely dynamic (collisional) quenching and static (ground-state complex formation) quenching between fluorophores and quenchers. In the simplest case of collisional

quenching, the dependence of the emission intensity on the quencher concentration (Q) is given by the well-known Stern-Volmer equation (Equation (1)). In this equation, I_0 and I represent the fluorescence intensities observed in the absence and presence of the quencher, respectively, and K_{sv} is the Stern-Volmer quenching constant, defining the quenching efficiency and which is related to the bimolecular collision process.

$$\frac{I_0}{I} = 1 + K_{SV}\ Q \qquad \text{(eq. 1)}$$

The linearity of Stern-Volmer plot (I_0/I) *versus* the quencher concentration (Q) denotes that one type of quenching mechanism is prevailing. Positive deviations from linearity of Stern-Volmer plots (concave with respect to the Y axis) suggest simultaneous dynamic and static extinction. The static quenching involves the formation of a nonfluorescent complex between the fluorophore and quencher in the ground state or the presence of a quenching entity that diminishes the amount of fluorescence molecules.[20,21]

Data collected by fluorescence emission spectra were used to plot the Stern-Volmer representations and to obtain linear regressions according to Equation (1) (Table 1) and to characterize the quenching process. In Figs. 3 and 4 are inserted the Stern-Volmer plots of the quenching of tryptophan fluorescence in copolymer PATrpF by picric acid and *o*-nitrophenol, respectively. Fig. 5 displays as insets the Stern-Volmer plots of the quenching of tryptophan fluorescence in copolymer PATrp by picric acid and *o*-nitrophenol, respectively.

As observed in Table 1, the values of K_{SV} are higher for fluorescence quenching of the two copolymers with *o*-nitrophenol. At the same time, the Stern-Volmer plot for fluorescence quenching with *o*-nitrophenol had a steeper slope than that corresponding to fluorescence quenching with picric acid (Fig. 5). The quenching efficiency, defined as $\eta = [(I_0 - I)/ I_0]$ x100 (%), was estimated to be 68 and 54 % for the two polymers in the case of picric acid and 87 and 83 % for PATrp and PATrpF, respectively, when *o*-nitrophenol was used as quencher (Table 1). As seen in Table 1, all the linear regression coefficients (r^2) were between 0.93 and 0.99, which suggests a high linear correlation. This linear correlation between the quencher concentration Q and I_0/I is in accordance with the Stern-Volmer equation. It can be concluded that *o*-nitrophenol is a more sensitive quenching agent than picric acid tested for the copolymers having tryptophan fluorophore in their structures.

EXPERIMENTAL

Materials. *L*-Tryptophan, acryloyl chloride, 1,1'-azobis(cyclohexanecarbonitrile), acrylic acid, itaconic acid, picric acid, *o*-nitrophenol, 1,4-dioxane, *N,N*-dimethylformamide (DMF), *L*-tryptophan methyl ester hydrochloride (TrpME) were used as received (Sigma-Aldrich).

Synthesis of N-acryloyl-L-tryptophan (**ATrp**): The monomer was prepared by a Schotten-Baumann reaction of *L*-tryptophan with acryloyl chloride (1.2 equivalents) in the presence of NaOH (2 equivalents).[19] To an aqueous solution of NaOH (2.0 g NaOH in 40 mL distilled water) was added *L*-tryptophan (5.10 g, 25 mmol) and the resulted solution was chilled to 0 °C by immersing in an external ice bath. Acryloyl chloride (2.42 mL, 30 mmol) was added dropwise at 0 ºC and the mixture was stirred for 2 h while the temperature was allowed to rise to room temperature. The mixture was acidified to pH=2-3 with concentrated HCl, and the precipitated product was filtered with suction, washed with water, and dried under vacuum at room temperature to afford an off white solid (yield 4.2 g, 65%). Anal. Calcd. for $C_{14}H_{13}N_2O_3$: C, 65.1; H, 5.5; N, 10.8. Found: C, 64.78; H, 5.48; N, 10.69. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 3.19-3.24 (dd, 2H, -CH_2CHCOOH), 4.54-4.60 (t, 1H, -C*H*COOH), 5.57-5.60 (dd, 1H, CH_2=C-, *cis*), 6.04-6.09 (dd, 1H, CH_2=C-, *trans*), 6.28-6.35 (dd, 1H, C=CH-), 6.96-7.14 (m, 3H, indole ring), 7.25-7.35 (d, 1H, indole ring), 7.53-7.60 (d, 1H, indole ring), 8.34-8.43 (s, 1H, -NH-CO-), 10.96 (s, 1H, NH- from indole ring), 12.62-13.74 (br, 1H, COOH). ^{13}C NMR (100 MHz, DMSO-d_6), δ (ppm): 27.6 (-CH_2CHCOOH), 54.4 (-NHCHCOOH), 109.3-112.4 (indole ring), 118.2-127.8 (indole ring and -CH=CH_2), 164.8 (-C=O), 174.3 (-COOH). IR (KBr), ν_{max} (cm^{-1}): 3414 (amide N-H), 3354 (OH from COOH), 3060-3000 (aromatic CH, broad), 2916 (aliphatic C-H), 2570 (O-H acid), 1714 (amide C=O), 1651 (aliphatic double bond and amide C=O), 1598, 1526 (aromatic ring), 1456 (C-N amide), 1221 (acyl O=C-O), 1113 (C-N amine), 975 (C=C, *trans*), 811 (=C–H).

Synthesis of the ternary copolymer poly(AA-co-IA-co-ATrp) (**PATrp**): The polymerization was carried out with 1,1'-azobis(cyclohexanecarbonitrile) (0.2% with respect to the amount of monomers) as an initiator in a degassed sealed tube.[23] Because oxygen gas retards free radical polymerizations, removal of the dissolved oxygen from monomer solutions was achieved simply by sparging a chemically inert gas. Acrylic acid (3.65 mL, 53.2 mmol), itaconic acid (1.73 g, 13.3 mmol), *N*-acryloyl-*L*-tryptophan (3.44 g, 13.3 mmol), 1,1'-azobis(cyclohexanecarbonitrile) (0.018 g) and 1,4-dioxane (40 mL) were placed in a dry glass ampoule equipped with a magnetic stirring bar, and then the solution was degassed with nitrogen. After the ampoule was flame sealed under vacuum, it was stirred at 80 °C for 72 h. The solution became viscous and its color changed from yellow to brown after 24 h. The copolymer was precipitated into a large excess of diethyl ether and a yellow powder was obtained after drying at 60 °C for 12 h.

Synthesis of the binary copolymer poly(AA-co-IA): Acrylic acid and itaconic acid were copolymerized in the 4:1 molar

ratio following the same procedure as above for the ternary copolymer. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 1.5-2.2 (CH_2 and CH, backbone) and 2.5-2.58 (CH_2, itaconic acid). IR (KBr), ν_{max}: 3500-3200 cm^{-1} (O-H), 2970-2930 cm^{-1} (C-H), 1720 cm^{-1} (C=O), 1455 cm^{-1} (C-H), 1425-1420 cm^{-1} (C=O) and 1190 cm^{-1} (C-H).[24]

Synthesis of copolymer poly(AA-co-IA) with pendant tryptophan groups (**PATrpF**): The amount of *N,N'*-dicyclohexylcarbodiimide (DCC) and *L*-tryptophan methyl ester hydrochloride (TrpME) introduced in reaction represents 10% in weight *versus* total COOH equivalents contained in the mass of polymer. To a solution of copolymer (4 g) in DMF (25 mL) a solution of DCC (1.174 g, 5.7 mmol) in DMF (5 mL) was added under stirring. After 1h, a solution of TrpME (1.452 g, 5.7 mmol) and triethylamine (5.7 mmol, 0.80 mL) in DMF (5 mL) was added. During the addition of the activating agent (DCC) and functionalization reagent (TrpME), the reaction flask was kept in a cold water bath. The reaction mixture was stirred for 24 h, at room temperature and for 2 h at 32 °C, in a dry nitrogen atmosphere. The dicyclohexylurea formed was filtered off. The solution was poured into a large amount of diethyl ether, and the polymer precipitated. The crude product was separated by decantation and dried in vacuum for 12 h. ^{1}H NMR (D_2O), δ (ppm): 1.67 (m, 5H, CH_2 from acrylic acid and backbone itaconic acid and CH tryptophan), 1.85 (broad, CH_2 from tryptophan), 2.28-2.42 (broad, CH, acrylic acid), 2.68-2.71 (2H, CH_2 from itaconic acid side chain), 3.85 (s, 3 H, OMe), 4.46 (s, 1H, CH-N, tryptophan chain), 7.19-7.62 (m, indole ring).

Measurements. The structure of all synthesized compounds was verified by ^{1}H, ^{13}C NMR, FTIR and UV-vis spectroscopy. The NMR spectra were recorded in DMSO-d_6 and deuterium oxide (D_2O) at room temperature on a Bruker Avance DRX 400 spectrometer with TMS as an internal standard. The all-chemical shifts were expressed in parts per million (ppm, δ) values. Fourier transform infrared (FTIR) spectra were run on a Bruker Vertex 70 spectrometer, in the 400-4000 cm^{-1} region, 64 scans, at room temperature, on KBr pellets. The UV light absorption spectra were measured in DMF, in quartz cells with Specord 200 Analytik Jena spectrophotometer in the spectral range of 250-500 nm. The fluorescence emission intensity measurements were carried out by a steady-state method in a 5-mm path length quartz cuvette with maximum volume of 2.5 mL at room temperature in DMF and dioxane, on a Perkin-Elmer LS 55 spectrofluorimeter. The quenching study was achieved using *o*-nitrophenol and picric acid as quenchers and an excitation wavelength of 280 nm.

CONCLUSIONS

Two *L*-tryptophan-based copolyacrylates were synthesized by a free radical polymerization and characterized through FT-IR, ^{1}H NMR, UV-vis absorption and emission spectroscopies. The fluorescence of the resulted copolymers recorded in DMF or dioxane solutions indicated that the presence of *o*-nitrophenol and picric acid led to changes in the microenvironment around tryptophan pendant groups that favoured their interaction. For both copolymers, *o*-nitrophenol acts as an efficient quenching agent, its detection limit being of 8×10^{-7} M for PATrpF and of 3.2×10^{-6} M for PATrp. The quenching pattern of the two tryptophan-containing copolyacrylates is similar in DMF and 1,4-dioxane.

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