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Novel water-soluble fluorescent polymer containing recognition units: Synthesis and interactions with PC12 cell

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Abstract

New water-soluble fluorescent poly(*N*-vinylpyrrolidone) (PVP) containing carbonylhydrazide recognition units was synthesized by free radical polymerization of *N*-vinylpyrrolidone in the presence of mercaptoacetic acid as chain transfer agent and then being modified by 1-pyrenebutyric acid hydrazide. FT-IR, ^{1}H NMR, gel permeation chromatography-multi-angle laser light scattering and fluorescence spectroscopy were used to characterize these polymers. Results of fluorescence measurements show that these polymers have a good affinity for deoxyribonucleic acid (DNA). The interactions with PC12 cell results indicated that the polymer with suitable molecular weight could penetrate into PC12 cell and emit fluorescence. This water-soluble polymer with recognition units and high luminescence can be used as a promising fluorescent probe for measurements of biomacromolecules and cells.

Keywords: Poly(*N*-vinylpyrrolidone); PC12 cell; DNA; Recognition; Fluorescence

1. Introduction

Fluorescence detection has been widely used as a versatile tool in analytical chemistry, biochemistry, cell biology, etc. [1]. During the past decade, research on polymers combined with fluorophore has attracted increasing interests because of the following two main reasons: (1) photochemical processes can be greatly modulated owing to microenvironmental effects, and (2) the chromophores can act as "reporters" to provide useful information on the conformation and dynamic properties of the polymers [2]. Many investigators have used fluorescent labeled water-soluble polymers to study phase separation [3], latex film formation [4], self-aggregation [5–7], electron transfer phenomena [8–10] and photoredox reaction [11]. A further challenge in this area is specific targeting, which would allow for the recognition and binding of the fluorescent polymer host to the target site and the chromophores act as "reporters" to provide useful information on the process of recognition.

As we all know, control of macromolecular structure and function through specific noncovalent interactions is

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central to a wide variety of applications in bio- and nanotechnology [12–14]. Embedding the recognition units within a polymer chain can potentially provide biologically inspired catalysts and delivery systems [15]. In recent studies, the incorporation of specific recognition elements has been employed in random copolymers [16,17], block copolymers [18], polyamines [19] and polymers containing single recognition element [15]. For our work, we have designed and synthesized water-soluble fluorescent polymers containing carbonylhydrazide recognition units by free radical polymerizations of *N*-vinylpyrrolidone in the presence of mercaptoacetic acid as chain transfer agent, then modified by 1-pyrenebutyric acid hydrazide. In these polymers, PVP was selected as the main chain of the polymers because of its excellent water solubility and biocompatibility [20], and pyrene was chosen as fluorescent chromophores for the following advantages: pyrene has a long singlet lifetime, can readily form excimers, acts as an energy acceptor via nonradiative energy transfer from several dyes and the vibronic band structure of its emission is sensitive to the environment [21].

In this study, binding efficiencies were investigated by the fluorescence emission changes of polymer when deoxyribonucleic acid (DNA) was added into the polymer–tris–HCl buffer solution at room temperature. Results showed that the polymer has a good affinity for DNA. To investigate the binding site between polymer and DNA, the fluorescence emission changes of 1-pyrenebutylric acid hydrazide (PyHy) and methyl 1-pyrenebutyrate were also measured when DNA was added into their tris–HCl buffer solutions at room temperature, respectively. Results illustrated that carbonylhydrazide groups are the recognition units. The interaction with PC12 cell results indicated that the polymer with suitable molecular weight could penetrate into PC12 cell and emit fluorescence. This water-soluble polymer with recognition units and high luminescence can be used as a promising fluorescent probe for measurements of biomacromolecules and cells.

2. Experimental

2.1. Apparatus

Steady-state fluorescence spectra were obtained on a Shimadzu RF-5301PC spectrometer (Japan). Melting points were measured on a Reichert 7905 melting-point apparatus (uncorrected). The infrared spectra were performed on a Nicolet 670 FT-IR spectro-photometer (USA). The mass spectra were recorded on a ZAB-HF-3F spectrometer (England). Elemental analysis was determined by a Flash 1112 series elemental autoanalyzer (Italy). ^{1}H NMR spectra were recorded on a Varian Mercury VX-300 MHz spectrometer (USA). Axiovert 200M inverse fluorescence microscope (ZEISS Co., Germany) and AxioCam HRO type digital camera (ZEISS Co., Germany) were used for observing and recording the morphology of PC12 cell. A DF-801 PH meter (Zhongshan University, China) was used.

2.2. Reagents

N,*N*′-dicyclohexyl carbodiimide (DCC), mercaptoacetic acid (MA) and *N*-vinylpyrrolidone (NVP) were all obtained from Acros Co.; 1-pyrenebutyl acid was purchased from Aldrich Co.; Herring Sperm DNA was obtained from Sigma Co.; tris(hydroxymethyl)aminomethane, 85% hydrazine hydrate and thionly chloride were purchased from Shanghai Chemical Reagents Co. China Center of Type Culture Collection (CCTCC) supplied PC12 cells. All solvents were of analytical grade and used as received. All solutions were prepared with doubly distilled water. NVP was purified by distillation under reduced pressure to remove the inhibitors before use. Azobis(isobutyronitrile) (AIBN) was recrystallized from methanol: mp 103 °C. Thionly chloride was purified by distillation before use. Methanol was dried and distilled before use according to standard procedures. Tris–HCl buffers (pH = 7.4) were prepared by mixing 0.05 mol/l of tris(hydroxymethyl)aminomethane aqueous solution with 1.0 mol/l HCl to the required pH value.

2.3. Synthesis

2.3.1. Synthesis of methyl 1-pyrenebutyrate

Thionly chloride (4.5 ml, 0.061 mol) was added dropwise to methanol (50 ml, 1.52 mol) below 0 °C, and at the end of addition 1-pyrenebutyric acid (1.5 g, 0.005 mol) was added. The mixture solution was stirred overnight at room temperature and refluxed for 2 h, then cooled to room temperature. The solvent was removed under reduced pressure. The crude products were purified by recrystallization from methanol to give methyl 1-pyrenebutyrate as yellowy crystals, 1.2 g, yield: 80%; mp: 46–48 °C; ^{1}H NMR ($CDCl_3$, δ ppm): 2.19 (m, 2H, $CH_2\mathbf{CH_2}CH_2$), 2.46 (t, 2H, $CH_2\mathbf{CH_2}CO$), 3.38 (t, 2H, $\mathbf{PyCH_2}CH_2$), 3.68 (s, 3H, $O\mathbf{CH_3}$), 7.8–8.2 (m, 9H, Py**H**).

2.3.2. Synthesis of 1-pyrenebutylric acid hydrazide (PyHy)

A mixture of methyl 1-pyrenebutyrate (1.2 g, 4 mmol), 85% hydrazine hydrate (15.0 g, 255 mmol) in 20 ml ethanol was stirred and refluxed for 1 h. Then the resulting mixture was cooled to room temperature. The solvent and excess hydrazine hydrates were evaporated under reduced pressure. The crude products were recrystallized from a mixture of ethanol/H_2O to give 1-pyrenebutylhydrazide as yellowy crystals, 0.72 g, yield: 60%; mp: 168–170 °C; ^{1}H NMR ($CDCl_3$, δ ppm): 2.16

(m, 4H, C**H**$_2$C**H**$_2$CO), 3.31 (t, 2H, Py**CH**$_2$), 3.80 (s, 2H, NH**NH**$_2$), 6.54 (s, 1H, **NH**NH$_2$), 7.8–8.2 (m, 9H, Py**H**). IR (KBr pellet, cm^{-1}): 3321.0 (s), 3194.4 (w), 3038.5 (m), 2938.8 (m), 2859.7 (w), 1641.7 (s), 1628.8 (s), 1429.3 (w), 997.8 (m), 845.1 (s) cm^{-1}. FAB-MS *m*/*z* (RI): 302 (100, M$^+$), 271 (41, M$^+$ −31, M–NHNH$_2$), 215 (69, M$^+$ −87, M–CH$_2$CH$_2$CONHNH$_2$); elemental analysis: Calcd. for $C_{20}H_{18}N_2O$ (%): C 79.37, H 5.95, N 9.26. Found (%): C 79.28, H 5.93, N 10.75.

2.3.3. Synthesis of PVP with single terminus carboxylic group (PVP–COOH)

NVP, AIBN and MA were dissolved in dioxane and purged with high pure nitrogen for 15 min, then it was stirred and heated to 60 °C for 3 h under nitrogen atmosphere. After cooled to room temperature, the polymers with single terminus carboxylic group (PVP–COOH) were recovered by precipitation in five-fold diethyl ether. PVP–COOH was purified by repeated reprecipitation from dioxane into diethyl ether for three times, and dried in vacuum until a constant weight.

2.3.4. Synthesis of fluorescent polymer containing recognition units (PVP–PyHy)

The solution of PVP–COOH in dioxane was supplemented with an excess of DCC in an equal volume of the same solvent. The mixture was stirred at 0 °C for 1 h with the addition of excess of 1-pyrenebutyric acid hydrazide in isopropanol. The solution was stirred overnight at 60 °C. Resulting polymer was precipitated into five-fold diethyl ether, purified by repeated reprecipitation from dioxane into diethyl ether for five times, and dried in vacuum until a constant weight.

2.4. Characterization of polymers

2.4.1. Analysis of end-carboxylic group

The number-average molecular weight (M_n) of PVP–COOH was determined by end-group analysis where the carboxyl groups of PVP–COOH were determined by direct titration with sodium hydroxide–ethanol solution and phenolphthalein was used as indicator. M_n was calculated from the following formula:

$$M_n = \frac{1000m}{c_{NaOH}(V_0 - V_t)},$$

where m (g) is the weight of PVP–COOH and c_{NaOH} (M) is the concentration of sodium hydroxide–ethanol solution, V_0 (ml) and V_t (ml) are the bulk of sodium hydroxide–ethanol solution before and after the titration, respectively.

2.4.2. Gel permeation chromatography-multi-angle laser light scatting

Gel permeation chromatography-multi-angle laser light scattering (GPC-MALLS) is convenient for determination of the true molecular weight and distribution of polymer without standard sample. Molecular weights (M_n), radii (R_n), and M_w/M_n of the samples were determined by a DAWN®DSP multi-angle laser photometer with a pump P100 (Thermo Separation Products, San Jose, USA) equipped with TSK-GEL G6000 PWXL with a G4000 PWXL column (7.8 mm × 300 mm) for aqueous solutions, and differential refractive index detector (RI-150) at 25 °C. The mobile phase was 0.5 M NaCl at a flow rate of 1.00 ml/min. Refractive index increments (d*n*/d*c*) were measured with a double-beam differential refractometer (DRM-1020, Otsuka Electronics Co.). Polymer concentrations for measurements were all about 2.0 mg/ml in 0.5 M NaCl, which were filtered with sand filter and 0.45 μm filter (CA, Puradisc TM 13 mm Syringe Filters, Whattman, England). Astra software was utilized for data acquisition and analysis.

2.4.3. ^{1}H NMR measurements

^{1}H NMR spectra were recorded on a Varian Mercury VX-300 MHz spectrometer operating at a proton frequency of 300 MHz. Samples (5–10 mg/ml) were dissolved in $CDCl_3$.

2.4.4. FT-IR measurements

FT-IR (KBr pellet) spectra were recorded on a Nicolet 670 FT-IR spectrophotometer (USA).

2.5. Fluorometric measurements

2.5.1. PVP–PyHy aqueous solution

The PVP–PyHy stock solution was prepared by individually dissolving 50 mg of PVP–PyHy I, II and III into distilled water to give a concentration of 5 mg/ml and further diluted to 0.25 mg/ml. Steady-state fluorescence spectra of PVP–PyHy were obtained at room temperature. The emission and excitation slit widths were 1.5 and 3.0 nm, respectively. The emission spectra of PVP–PyHy I, II and III were all measured in the range of 350–500 nm excited at 342 nm.

2.5.2. Interactions of PVP–PyHy with DNA

To observe the binding efficiencies between PVP–PyHy and DNA, PVP–PyHy I was selected as the experimental sample. The PVP–PyHy I stock solution was prepared by dissolving 50 mg of PVP–PyHy I into tris–HCl buffers to give a concentration of 5 mg/ml and further diluted to 0.1 mg/ml. DNA stock solution was prepared by dissolving 100 mg of Herring Sperm DNA into tris–HCl buffers to give a concentration of 10 mg/ml and further diluted to 1 mg/ml. Steady-state fluorescence spectra of PVP–PyHy I were recorded at room temperature. The emission and excitation slit widths were both 3.0 nm. The emission spectra of PVP–PyHy I when adding DNA into PVP–PyHy I solution were measured in the range of 350–500 nm excited at 342 nm.

2.5.3. Interactions of PyHy or methyl 1-pyrenebutyrate with DNA

When DNA was added, the fluorescence intensity changes of PyHy and methyl 1-pyrenebutyrate were investigated in tris–HCl buffers. The general scheme is as follows: DNA stock solution was prepared as described in Section 2.5.2. Twenty microliter $CHCl_3$ solution of PyHy (as probe) and methyl 1-pyrenebutyrate (as probe) was added to two vials and the solvent was evaporated in vacuum, respectively. Tris–HCl buffer (5.0 ml) was added to these vials. It should be noted that all the aqueous solutions contained excess of PyHy and methyl 1-pyrenebutyrate residues at the same concentration of 4.0×10^{-6} M. The solutions were allowed to equilibrate for 24 h prior to fluorescence runs. The emission spectra of PyHy and methyl 1-pyrenebutyrate as DNA added were measured both in the range of 350–500 nm with excitation at 342 nm. The emission and excitation slit widths were 5.0 nm and 10.0 nm, respectively.

2.6. Interactions of PVP–HyPy with PC12 cell

The balanced salt aqueous solution is composed of 140 mmol/l NaCl, 2 mmol/l $CaCl_2$, 4.2 mmol/l KCl, 0.7 mmol/l $MgCl_2$, 1 mmol/l NaH_2PO_4, and 10 mmol/l HEPES, which were filtered with 0.22 μm filter before use. Firstly, the morphology of the PC12 cells in growth medium solution was taken photos in bright field and fluorescence at the excitation wavelength of 360 nm, respectively.

Secondly, 100 μl growth medium solution containing PC12 cells were centrifugalized at the condition of 25 °C and 1000 rpm/min, and the top solution was discarded. Twenty microliter polymer solution (5 mg/ml) was added to the residue of growth medium solution, and 80 μl balanced salt aqueous solution was again added after 5 min, then taken out 5 μl solution from the mixture solution to take photos in bright field and in fluorescence at the excitation wavelength of 360 nm, respectively.

Thirdly, the mixture solutions were centrifugalized and the top solution was discarded, added 100 μl balanced salt aqueous solution and 5 μl mixture solution was taken out to take photos in bright field and in fluorescence at the excitation wavelength of 360 nm, respectively. Repeat this process again.

3. Results and discussion

3.1. Synthesis and characterization of PVP–COOH

PVP–COOH was prepared by free radial polymerization of NVP in dioxane with different amount of MA as chain-transferring agents. In the synthesis of PVP–COOH, the ability of mercaptans to transfer the chain during the radical attack provides the carboxyl functional group onto a single terminus of a polymeric chain. The M_n of PVP–COOH was determined by the determination of carboxyl groups by direct acid–base titration method. And as expected the polymers molecular weight depended on the concentration of the corresponding mercaptoacetic acid and decreased with increasing the amount of mercaptoacetic acid added to the system. The characterizations of PVP–COOH with different M_n are summarized in Table 1.

The chemical structure of PVP–COOH was characterized by FT-IR. IR spectra verified the existence of the stretching vibration of **CO**OH and OH bond in PVP–COOH appears at 1715 and 3448 cm^{-1}, respectively.

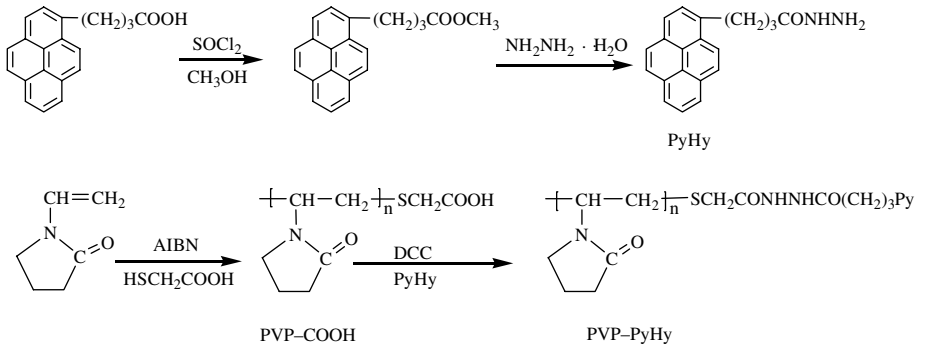
3.2. Synthesis and characterization of PVP–PyHy

Three corresponding kinds of PVP–PyHy were individually synthesized by coupling PVP–COOH I, II and III with excess of PyHy in the presence of DCC as dehydration agent, purified by repeated reprecipitation from dioxane into diethyl ether. These polymers can all be easily dissolved in water. The synthesis procedure of PyHy and PVP–PyHy are illustrated in Scheme 1.

Table 1
Characterization of PVP–COOH and PVP–PyHy

Polymer		MA/NVP[a]	M_n (10^4 g/mol)	M_w (10^4 g/mol)[b]	M_w/M_n[b]
PVP–COOH	I	0.05	2.13[c]		
	II	0.0054	3.62[c]		
	III	0.0018	5.56[c]		
PVP–PyHy	I		2.003[b]	4.006	2.00
	II		3.528[b]	9.314	2.64
	III		5.005[b]	9.709	1.94

[a] Molar ratio of MA to NVP in the feed.
[b] From GPC-MALLS analysis.
[c] From end-group analysis.



Scheme 1. Schemes for preparation of PyHy and PVP–PyHy.

Resulting PVP–PyHy were characterized by FT-IR and ^{1}H NMR. The peak at 1715 cm^{-1} which is ascribed to the stretching vibration of C=O in **CO**OH disappears after the coupling of PVP–COOH with PyHy. The stretching vibration of C=O on PVP ring and **CO**NH of PVP–PyHy both appear at 1655 cm^{-1} since the **CO**OH group has changed into **CO**NH group as that of the PVP ring. This showed that PyHy had successfully conjugated with PVP–COOH.

The ^{1}H NMR spectrum of PVP–PyHy II in $CDCl_3$ is shown in Fig. 1. The ^{1}H NMR spectrum of PVP–PyHy I and III in $CDCl_3$ is similar to PVP–PyHy II spectrum (not shown). The proton signals of pyrene in PVP–PyHy appear at the chemical shifts (7.8–8.2 ppm). This confirmed that PyHy had successfully conjugated with PVP–COOH. Signals between 1.1 and 3.9 ppm are assigned to the saturated protons of the CH_2, CH and NH groups.

The molecular weights of the PVP–PyHy were determined by GPC-MALLS and the results were listed in Table 1. The M_n of the PVP–PyHy should be larger than that of the corresponding PVP–COOH due to the coupling of PyHy with PVP–COOH while the GPC-MALLS results give a little less value. This may be explained by the fact that the technique of end-group analysis becomes insensitive at high molecular weights, as the fraction of end groups becomes too small to be precisely measured.

3.3. Fluorometric measurements

3.3.1. PVP–PyHy aqueous solution

The fluorescence emission spectra of PVP–PyHy I, II and III aqueous solution at the same concentration of 0.25 mg/ml are presented in Fig. 2. It is shown that the fluorescent emission intensity of PVP–PyHy decreased with increasing M_n of the PVP–PyHy at the same concentration, which is resulted from the high concentration of pyrene groups in PVP–PyHy with small M_n. No excimer (around 480 nm) formation was observed in Fig. 2 under that concentration because the effective concentration of pyrene in these cases was less than 10^{-5} M, well below the minimum concentration required for excimer formation that involves free pyrene [22]. The PVP–PyHy polymers have strong fluorescence in aqueous solution, which may be promising for use as water-soluble fluorescence probe.

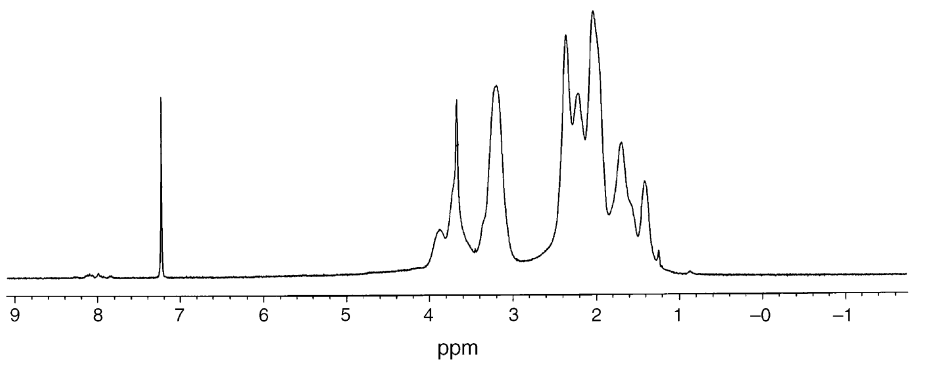


Fig. 1. ^{1}H NMR spectrum of PVP–PyHy II in $CDCl_3$.

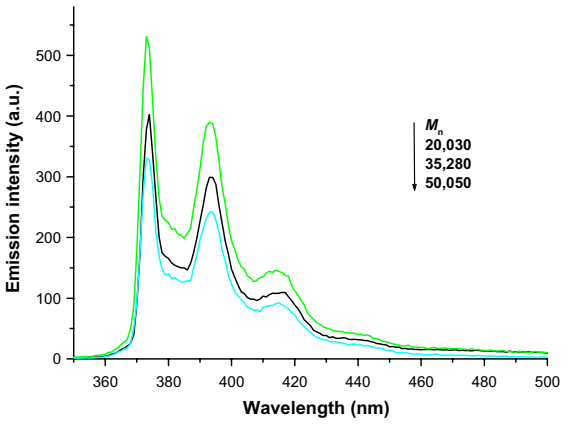


Fig. 2. Fluorescent emission spectra of PVP–PyHy I, II and III aqueous solution at the same concentration of 0.25 mg/ml, λ_{ex} = 342 nm.

3.3.2. Interactions of PVP–PyHy with DNA

The affinity of the polymeric hosts for DNA was experimentally quantified via fluorescence titration in tris–HCl buffers. PVP–PyHy I has characteristic monomer emission peak at 376 and 396 nm in tris–HCl buffers. The peak at 376 nm was selected to observe upon addition of DNA.

In this study, the fluorescence intensity changes due to volume increasing could be almost neglected as only 0.012 ml DNA solution added into 3.5 ml PVP–PyHy I solution. Polymeric fluorescence emission intensity changes in tris–HCl buffers as DNA addition are illustrated in Fig. 3. Fluorescence intensity of the PVP–PyHy I solution decreased upon DNA addition. The fluorescence quenching illustrated that interactions had happened between PVP–PyHy I and DNA. The fluorescence intensity changes at 376 nm are 25 and 46 upon addition of 0.002 and above 0.01 mg of DNA, respectively. As the addition of more than 0.01 mg of DNA, the fluorescence intensity changes almost keep constant, showing that the interactions between PVP–PyHy I and DNA will come to balance. While there were no much changes in relative fluorescence intensity during the titration, presumably for the steric hindrance of the bulky chain [15].

3.3.3. PyHy and methyl 1-pyrenebutyrate with DNA addition

To determine the binding sites between polymer and DNA, we also measured the fluorescence emission changes of 1-pyrenebutylric acid hydrazide (PyHy) (Fig. 4) and methyl 1-pyrenebutyrate (Fig. 5) when DNA was added into their tris–HCl buffer solution at room temperature, respectively.

Fig. 4 showed that fluorescence emission intensity of PyHy decreased with increasing amount of DNA added into tris–HCl buffers. The fluorescence quenching illustrated the interactions had happened between PyHy and DNA. While there was no fluorescence quenching observed upon DNA addition into methyl 1-pyrenebutyrate tris–HCl buffer solution (illustrated in Fig. 5), which demonstrated that there were no affinity between methyl 1-pyrenebutyrate and DNA. That the emission intensity of methyl 1-pyrenebutyrate increased a little may be resulted from the better solubility of the hydrophobic probe as DNA added. The carbonylhydrazide group of PyHy is the only difference between structures of PyHy and methyl 1-pyrenebutyrate. Then, we can conclude that affinity between PVP–PyHy and DNA was resulted from the carbonylhydrazide groups of PyHy.

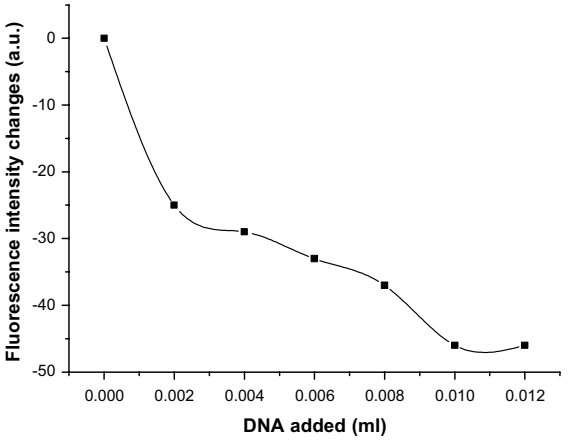


Fig. 3. The fluorescence emission changes of PVP–PyHy I at 376 nm upon addition of DNA (1 mg/ml) at room temperature. Concentration of PVP–PyHy I is 0.1 mg/ml, λ_{ex} = 342 nm.

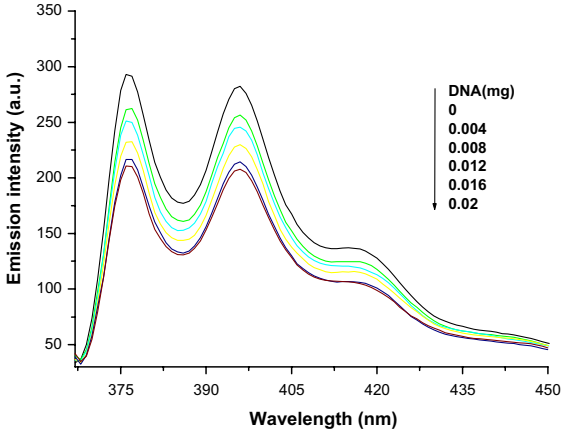


Fig. 4. Fluorescence emission spectra of PyHy as DNA added. λ_{ex} = 342 nm.

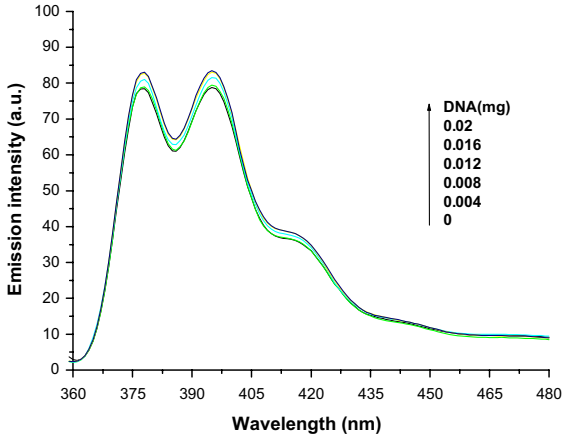


Fig. 5. Fluorescence emission spectra of methyl 1-pyrenebutyrate as DNA added. λ_{ex} = 342 nm.

3.4. Interactions with PC12 cell

In order to test the potential application of these water-soluble polymers as probes in biomacromolecules and cells, we used PVP–PyHy I and PVP–PyHy III to test PC12 cell. The photos of PC12 cell are listed in Fig. 6. (a) and (b) are the images of PC12 cells in bright field and fluorescence in aqueous solution without polymer, respectively. The images of PC12 cells whose growth medium solutions were added 20 μl PVP–PyHy I aqueous solution (5 mg/ml) and 80 μl balanced salt aqueous solution was again added after 5 min, and then centrifugalized, are shown in (c) and (d) in the bright field and in fluorescence, respectively. (e)–(h) are the images of PC12 cells in the bright field and fluorescence after being washed and suspended by balanced salt solution for the first (e, f) and second time (g, h). These photos showed that PC12 cells have perfect morphology. We concluded that the PVP–PyHy I penetrated into the cells and emitted fluorescence. While for the PVP–PyHy III aqueous solution (5 mg/ml), there were no cells images appearing after treating residual solution for the first time in the fluorescence (as figured in (l)). Under the same experimental conditions, the results showed that the penetrability of PVP–PyHy to PC12 cells is relevant with its molecular weight. This may be explained by higher steric hindrance of the bulky chain of polymer arising from high molecular weight.

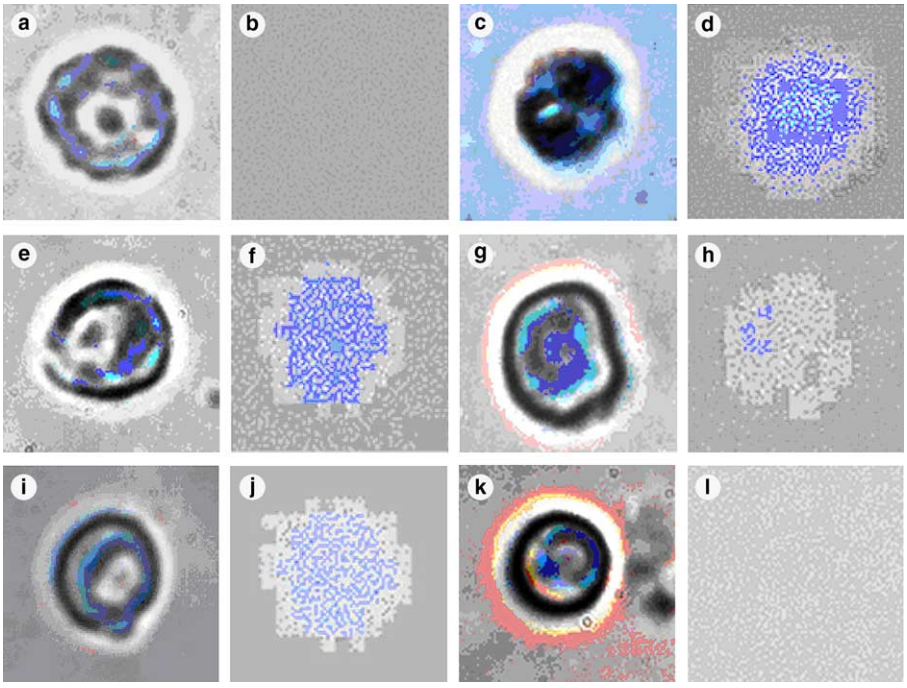


Fig. 6. Photos of PC12 cells in the bright field and fluorescence. (a), (b): PC12 cells in bright field and fluorescence in aqueous solution without polymer; (c), (d): PC12 cells in bright field and fluorescence after the addition of 20 μl PVP–PyHy I aqueous solution; (e)–(h): PC12 cells in bright field and fluorescence after being washed and suspended by balanced salt solution for the first and second time; (i), (j): PC12 cells in bright field and fluorescence after the addition of 20 μl PVP–PyHy III aqueous solution; (k), (l): PC12 cells in bright field and fluorescence after being washed and suspended by balanced salt solution for the first time.

4. Conclusion

New water-soluble fluorescent poly(*N*-vinylpyrrolidone) (PVP) containing the carbonylhydrazide recognition units were synthesized by free radical polymerization, and then modified by 1-pyrenebutyric acid hydrazide. Fluorescent results showed that the polymers have a good affinity for DNA. The changes of fluorescent emission intensity of PyHy and methyl 1-pyrenebutyrate when DNA was added illustrated that the carbonylhydrazide groups are recognition units. The interactions with PC12 cell results indicated that the polymer with suitable molecular weight could penetrate into PC12 cell and emit fluorescence. This water-soluble polymer with recognition units and high luminescence can be used as a promising fluorescent probe in the field of biomacromolecules and cell research.

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