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A differential approach towards understanding the enhanced emission induced superior bio-imaging and cytotoxicity within block copolymeric nanomicelles

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ABSTRACT

Two tri-block copolymers P123 ($PEO_{19}PPO_{69}PEO_{19}$) and F127 ($PEO_{100}PPO_{65}PEO_{100}$) have been employed to form polymeric nanomicelles and function as potential nanocarriers for an anticancer pyrazoline derivative (PYZ) in aqueous buffer solution for biological studies. Encapsulation within these nanomicelles considerably enhanced the fluorescence of the PYZ compared to its low fluorescence in aqueous buffer medium. The effect of the micellar structures on the photophysical properties of PYZ have been demonstrated by means of steady state and time resolved fluorescence spectroscopy. Variation in hydrophilicity of the corona region was found to be a prime factor in modulating the location of the PYZ within the micelles which in turn influenced its corresponding enhanced emission and cytotoxicity. These drug encapsulated nanomicelles were found to be successfully internalized into the MCF-7 cells to demonstrate high-quality fluorescent images. The location of PYZ within the polymeric micelles influenced the CAC (Critical Aggregation Concentration)/CMC (Critical Micellar Concentration) ratio which modulated their drug release capacity resulting in a variation in their cytotoxicity.

1. Introduction

The necessity to maximize the therapeutic effectiveness of drugs with minimal side-effects turned the spotlight on the amphiphilic block copolymer (BCP) systems. Amphiphilic block copolymers with a general formula, $PEO_x-PPO_y-PEO_x$ have two distinguished regions: a hydrophilic corona region composed of poly ethylene oxide (PEO) and a hydrophobic core composed of poly propylene oxide (PPO), which are known to self-assemble to form core-shell polymeric nanoscopic micelles with a narrow size distribution [1–4]. Their potentiality towards solubilizing water insoluble drugs without hydrolysis, enzymatic degradation, protein adsorption and cellular adhesion compels scientists to use them now days as the most promising polymeric nano drug delivery system (PNDDS) [5–7]. These PNDDS substantially decreases the risk of systemic toxicity, increases the extent of therapeutic effect by more active targeting to tumor cells with preferential accumulation in spite of easy clearance by reticuloendothelial system.They have also potential towards overcoming multidrug resistance of certain types of cancer cells and delivering multiple chemotherapeutic drugs and imaging agents together within the same polymeric drug delivery scaffold [8–10]. Among the various tri-block copolymers, P123 and F127 are the most extensively studied drug delivery systems due to their thermodynamic stability and enhanced drug loading capacity along with weak immunogenicity [11]. Micellar induced drug delivery systems require low critical micellar concentration (CMC) to rule out the possibility of induced toxicity and enhanced specific targeting with high bio-availability [12]. A mixed micelle composed of L61 and F127 loaded with doxorubicin have already reached Phase III stage [13]. Both the pluronics are able to convert themselves into non fluidic hydrogel which is very suitable for protein drug delivery as the polypeptides can remain concentrated *in vivo* without aggregation within the polymer matrix [14]. Pyrazoline derivatives (PYZ) are important nitrogen containing compounds and have wide range of biological activity including anti-inflammatory [15], antidepressant [16], anticancer [17], antibacterial, antitubercular [18] and analgesic [19] activities due to their pyrazole motif. Pyrazoline derivatives are also well established potential chemotherapeutic agents [20]. So far various pyrazoline derivatives have been recognized as inhibitors of

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cyclin-dependent kinase [21], heat shock proteins [22], vascular endothelium growth factors [23], and P-glycoprotein [24]. To date, a variety of fluorescent drugs and organic probes have been developed for *in vitro* and *in vivo* imaging and sensing. But their poor water solubility, photo degradation and inability to overcome the cellular transport barriers results in poor pharmacokinetic profiles, risk of systemic toxicity, low extent of dose delivery and disappointing therapeutic efficiency [3,25,26]. Therefore applying biodegradable polymer matrix having hydrophobic part to accommodate water insoluble drug or probe is considered as highly emerging nano capsule like polymeric nano drug delivery system (PNDDS) [27]. Normally therapeutic agents are linked to the polymeric system covalently but physical encapsulation has its own effectiveness towards releasing drugs with more efficacy and ease. The present investigation is aimed at designing a simple but unique BCP/PYZ couple based on the location dependent sensing ability within tri-block copolymers and to understand how the different locations of a single drug in block copolymers can modulate the enhanced cytotoxicity and imaging. In this study, block copolymer micelles acted as potential nanocarriers to encapsulate a pyrazoline derivative. The effect of spatial confinement on the emission properties was systematically envisaged by means of steady state and time resolved fluorescence spectroscopy. Temperature dependent time resolved response was also monitored to understand their different location in two different tri-block copolymers namely P123 and F127. Cytotoxicity studies and fluorescence images of the pyrazoline derivative encapsulated within polymer micelles internalized into MCF-7 cells demonstrated the potential of BCP/PYZ couple as fluorescent probes for superior bio-imaging and effective cytotoxic agent.

2. Experimental section

2.1. Materials

Pyrazoline derivative (PYZ)[($C_{32}H_{24}N_5Br$; MW: 556); {5-((4′S,5′R)-1′-(4-bromophenyl)-4′,5′-dihydro-4′,5′-diphenyl)-1H-pyrazol-3-yl-3-methyl-1 phenyl-1H-pyrazole-4-carbonitrile}] (Fig. S1) was synthesized following the procedure as described elsewhere [28]. Tri-block copolymer F127 and P123 were purchased from Sigma-Aldrich and were used as received. The water used in all the studies was treated with a Milli-Q water purification system. Disodium hydrogen phosphate (Na_2HPO_4) and Sodium dihydrogen phosphate (NaH_2PO_4) were acquired from E-Merck and was used as received. All other solvents were purchased from E Merck and were refluxed with calcium chloride and distilled before use. 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) were commercially available from Sigma-Aldrich.

2.2. Methods

2.2.1. UV–vis, steady state and time resolved fluorescence study

UV–vis absorption spectra were measured using a Shimadzu UV–vis 1700 spectrophotometer with a matched pair of silica cuvettes of path length 1 cm. Photoluminescence spectra of PYZ in different medium was acquired by a Shimadzu spectrofluorimeter (model RF5301) with a quartz cell of path length 1 cm.

The fluorescence decay time measurement was performed using a nanosecond diode laser at 370 nm (IBH, N-295) as the light source having a typical response time of 10 ps. The decays were analyzed using IBH DAS-6 decay analysis software. For all the lifetime measurements, the fluorescence decay curves were analyzed by a single and bi-exponential iterative fitting program provided by IBH such as

$$I(t) = \sum_{i-1}^{n} A_i e^{-t/\tau_i}$$

Where $I(t)$ is the intensity of the fluorescence at time t, A_i is the pre-exponential factor for the fraction of the fluorescence intensity, τ_i is the fluorescence lifetime of the emitting species, and n is the total number of emitting species. Average lifetimes for all the bi-exponential decays were calculated from the fluorescence decay curves using the following equation:

$$\tau_i = \frac{\alpha_1\tau_1 + \alpha_2\tau_2}{\alpha_1 + \alpha_2}$$

here, α_i is a pre-exponential factor representing the fractional contribution of the decaying component with a lifetime τ_i.

Time resolved fluorescence anisotropy studies were carried out by measuring the polarized fluorescence decays, $I_{\parallel}(t)$ and $I_{\perp}(t)$, where $I_{\parallel}(t)$ and $I_{\perp}(t)$are the decays for parallel and perpendicular polarizations, respectively, with respect to vertically polarized excitation light. From $I_{\parallel}(t)$ and $I_{\perp}(t)$decays, the anisotropy decay function $r(t)$ was constructed using the following relation:

$$r(t) = \frac{I_{\parallel}(t) - GI_{\perp}(t)}{I_{\parallel}(t) + 2GI_{\perp}(t)}$$

where G is a correction factor for the polarization bias of the detection setup. The G factor was independently determined by using the horizontally polarized excitation light and measuring the two perpendicularly polarized fluorescence decays with respect to the excitation polarization.

2.2.2. Preparation of solutions

Since both the polymers used in this study are highly water soluble and forms micelle at a very low concentration we opted direct physical entrapment or dissolution method for loading of PYZ within the micelles [29,30]. At first the PYZ stock solution (10^{-4} M) in acetonitrile was evaporated to dryness. Then requisite amount of polymer solutions were added in two different vials to make the final concentrations of the solutions as 0.1 mM and 2 mM (P123 and F127 respectively). The solutions were then agitated in a thermostatic bath for two days to ensure maximum loading of PYZ within the micelles. In this context it is worth mentioning that knowledge of aggregation number of both the polymers at the experimental temperature i.e., 25 °C is necessary [31].

2.2.3. Drug loading efficiency in micelles

Drug loading efficiency was monitored by measuring the concentration of PYZ in aqueous medium. The PYZ loaded polymeric micelles were first centrifuged for 20 min at a speed of 10000 rpm to remove the undissolved drug from the micelles. After obtaining the supernatant except the plaques, the aqueous phase was diluted at 1:5 ratio and the concentration of the free PYZ was measured by its emission maxima around 480 nm. We used unloaded micelles as control system. The loading efficiency was determined by the following equation

$$Loading\ \ Effeciency = \frac{Entrapped\ \ Drug\ with\ in\ \ \ the\ micelles}{Total\ amount\ \ of\ \ drug\ \ added} \times 100$$

2.2.4. Drug release studies

Release of PYZ from the block copolymer nanomicelles was monitored by dialysis procedure. At first the PYZ loaded polymeric micelles were transferred into a dialysis membrane tubing (Thermo, Slide-A Lyzer MINI Dialysis Devices, 3.5 K MWCO). The tubing was immersed into a phosphate buffer solution (PBS, 0.2 M,