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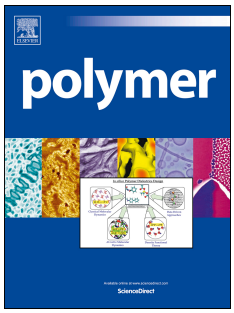

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FRET-based acrylic nanoparticles with dual-color photoswitchable properties in DU145 human prostate cancer cell line labeling

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Abstract

Dual-color photoswitchable fluorescent polymer nanoparticles have emerged as an important type of cell detectors. Here, dual-color acrylic nanoparticles containing spiropyran and azo-carbazole derivatives with remarkable photostability and photoreversibility were employed. These prominent properties were attributed to the covalent bonding between the incorporated chromophores and the polymeric matrix and provided efficient fluorescence resonance energy transfer phenomenon. The obtained nanoparticles were purified after dialysis and delivered into DU145 cancer cells. Trypan blue dye exclusion assays showed that these nanoparticles were biocompatible with no toxicity to the cells. Fluorescence microscopy images demonstrated that the prostate cancer cells containing nanoparticles exhibited excellent cell uptake with green and red fluorescence emissions after primary excitation at 410 nm and subsequent irradiation at 365 nm, respectively, depicting their dual-color characteristics.

Keywords: biocompatible; cell labeling; FRET; photoswitchable; nanoparticle.

1 Introduction

Dual-color photoswitchable fluorescent (DCPF) nanoparticles represent a new class of materials for exploitation of fluorescent technique in biological [1–3]. These fluorescent nanoparticles, based on fluorescence resonance energy transfer (FRET) phenomenon, have received many attentions because of some advantages over conventional fluorescent dyes as distinguishing fake positive signals by aberrant fluorescent biomolecules and targeted locations [4,5]. Hence, they have shown versatile applications in optically addressable devices [2], drug delivery [6], cell labeling and imaging [2,7,8], bioimaging and rewriteable pattering [9,10], sensing tools and optical switching [11–13].

Photoswitchable fluorescence labeling of cells and biomolecules has become one of the most important techniques to study molecular processes within biological systems. Utilization of the traditional fluorescence technologies have many restrictions practically, like autofluorescence or intrinsic false positive signals of biological systems that can generate interferences and consequently obscure required signals [4,5,7]. Photoswitchable polymer nanoparticles that modulate fluorescence with external light stimulation will be a good resolution here. In fact, reversible fluorescence modulation switches fluorophores “on” and “off” and would be exploited to distinguish fake signals of background from original ones [2]. In many researches, the fluorescent dye is doped or physically adsorbed into a polymeric matrix that raise potential dye leakage and dye aggregation, and consequently produces cytotoxicity for cells and blinking in long time. These represent additional drawbacks for their advanced biological, in-vitro and in-vivo applications [7,14]. To address these problems, dual-color photoswitchable fluorescent nanoparticle with covalent bonding between chromophores and polymeric matrix demonstrate several advantages over the traditional doped ones (without any chemical linkage). These advantages will donate

remarkably high brightness, extraordinary photostability, photoreversibility, high fatigue resistance and biocompatibility with potential application in living cells [15].

Dual-color polymer nanoparticles based on FRET consist of both donor and acceptor moieties. Photochromic compounds like spiropyrans are of high interest and act as acceptor chromophores [16,17]. Spiropyran derivatives respond to incident light and involve in a photoinduced ring-opening reaction that spiro (SP) form changes to merocyanine (MC) form with different spectroscopic properties [18–20]. Merocyanin isomer of spiropyran can receive energy from a fluorescent dye through fluorescence resonance energy transfer. Fluorescent molecules such as azo-carbazole ethyl acrylate (AzoCzEA) [15], boron-dipyrromethene phenol methacrylate (BDMPA) [9] and nitrobenzoxadizole (NBD) [14] can be used in these systems as donor moieties. To achieve complete energy transfer, fluorescence emission of the fluorescent molecule must have a prominent overlap with absorption band of the acceptor chromophore.

Polymer nanoparticles containing azo-derivatives have been extensively investigated due to their changes in shape, size, alignment, porosity and polarity [21–23]. These are the consequence of cis-trans isomerization induced by UV/Vis irradiation [24].

Zhu and coworkers [2] used photochromic spiropyran derivative and fluorescent perylene diimide to prepare dual-fluorescent polymer nanoparticles through emulsion polymerization. Then, they employed these photoreversible fluorescent nanoparticles as a tool for optically addressable living cells that exhibit photoreversible red-green fluorescence in the cytosolic media. Chen et al [9] reported the preparation of amphiphilic photoswitchable fluorescent nanoparticles using boron-dipyrromethene (BODIPY) and spiropyran-linked methacrylate (SPMA) by RAFT polymerization. They used chemical linkages for incorporation of the fluorescent dye to nanoparticles to improve their previous investigations [14,25] in which the fluorescent dye was doped to the nanoparticles.

This promoted photostability and photoswitchability for cell labeling and other biological applications.

The current research, based on a FRET system, focuses on the utilization of dual-color nanoparticles with covalent bond formation between all components that are excited at a single wavelength and emit at two higher wavelengths. Hence, photoswitchable probes containing AzoCzEA and SPEA were copolymerized with methyl methacrylate (MMA) through emulsion polymerization with respect to our previous report in which, they were fully-characterized and their spectral features were studied completely [15]. Here and in continuum, their photoreversibility and non-radiation energy transfer kinetics are explained. To show the potential applications, cytotoxicity and biocompatibility of the prepared nanoparticles are studied and in-vitro experiments displayed their spectacular dual-color responses in DU145 prostate cancer cells by fluorescence imaging.

2 Experimental section

2.1 Materials

2,3,3-trimethylindolenine and sodium dodecyl sulfate (SDS, 99%) were purchased from Sigma-Aldrich. All of the solvents, 2-hydroxy-5-nitrobenzaldehyd, methyl methacrylate (MMA), potassium persulfate (KPS) as the initiator, sodium bicarbonate as a buffer ($NaHCO_3$), carbazole (89%), sodium nitrite ($NaNO_2$), potassium hydroxide (KOH), hydrochloric acid (HCl, 37%), 4-nitro aniline, triethylamine, Triton X-100, 2-bromoethanol, acryloyl chloride (AC), sodium chloride, potassium dihydrogen phosphate, disodium hydrogen phosphate and EDTA were supplied by Merck Chemical Co. Trypan blue, phosphate buffer saline and formaldehyde were all purchased from Sigma-Aldrich. Tetrahydrofuran (THF) was dried over sodium and distilled off. Other

solvents and all reagents were used without further purification. Deionized (DI) water was used in the polymerization recipe.

2.2 Synthesize of SPEA chromophore

1′-(2-Acryloxyethyl)-3′, 3′-dimethyl-6-nitrospiro-(2-H-1-benzopyran-2,2′-indoline) (SPEA) was synthesized according to our previously reported procedure [18]. Typically, 1-(2-hydroxyethyl)-2,3,3-trimethyl-3*H*-indolium bromide was synthesized through a substitution nucleation reaction between 2,3,3- trimethylindolenine (1.3 g, 8 mmol) and 2-bromoethanol (1.23 g, 10 mmol) in 20 mL methyl ethyl ketone (MEK) under N_2 and refluxed for 24 h. Then the mixture was cooled down to room temperature to give pink precipitates, which were filtered off and washed with cold acetone. In continuum, 1-(2-hydroxyethyl)-2,3,3-trimethyl-3*H*-indolium bromide (1.46 g, 5 mmol) subjected to isomerization reaction with a solution of potassium hydroxide (0.46 g, 8 mmol) in water (40 mL). Thus, (R/S)-9,9,9a-trimethyl- 2,3,9,9a-tetrahydrooxazolo [3,2-a] indole (R/S) was extracted by diethyl ether (3×40 mL). Afterward, condensation reaction between the (R/S)-9,9,9a-trimethyl- 2,3,9,9a-tetrahydrooxazolo[3,2-a] indole (R/S) (0.43 g, 2 mmol) and 2-hydroxy-5-nitrobenzaldehyde (0.52 g, 3 mmol) was performed in ethanol (15 mL) to give (R/S)-2-(3′,3′-dimethyl-6-nitro-3′H-spiro- [chromene-2,2′-indole]-1′-yl)ethanol (R/S) (SP−OH). Modification reaction of SP−OH (2 g, 6 mmol) with acryloyl chloride (AC) (4.2 mL, 18 mmol,) was carried out in dry THF (20 mL) as the solvent by using of triethylamine (0.8 mL, 8 mmol) at 0 °C. Then, temperature of reaction reached to room temperature and the reaction continued for 8 h. Finally, yellow precipitates of SPEA were filtered and washed with cold acetone.

2.3 Synthesize of AzoCzEA chromophore

(4-Nitrophenyl)-[3-[N-[2- (acryloyloxy)ethyl]carbazolyl]] diazene (AzoCzEA) was synthesized according to our reported procedure [15]. At first, N-(2-hydroxyethyl) carbazole was synthesized by nucleation substitution reaction between carbazole (3.93 g, 23.5 mmol) and 2-bromoethanol (3.38 g, 27 mmol) in 40 mL dimethyl formamid (DMF) as the solvent in the presence of potassium hydroxide at room temperature for 12 h. Then, the obtained mixture was poured into distilled water and white solids were filtered, washed 3 times with water, air-dried and finally recrystallized from a 1:1 cyclohexane: toluene mixture to give white needle crystals (89% yield). To synthesize 3-[(4-Nitrophenyl)] azo-9H-carbazole-9-ethanol (NACzEtOH), 4-nitroaniline (0.28 g, 2 mmol) was dissolved in a solution of concentrated HCl (1 mL) in water (30 mL) at 0 °C. After dissolution, sodium nitrite (0.16 g, 2.2 mmol) was dissolved in 1 mL water and added dropwise to above solution while stirring for 20 min. Then 0.1 g SDS was added. Another solution containing N-(2-hydroxyethyl) carbazole (0.64 g, 3 mmol) in DCM (30 mL) was added to the aforementioned mixture at room temperature. After 24 h, 20 mL ethanol was added and two phases were separated. The dispersion was heated to remove the DCM layer and the red precipitate was filtered, washed with water and air dried. Crystallization was done in 1:1 solution of ethanol: chloroform to give red powder (0.73 g, 92% yield). Finally, (4-nitrophenyl)-[3- [N-[2- (acryloyloxy) ethyl] carbazolyl]] diazene (AzoCzEA) was synthesized through the reaction of NACzEtOH (0.72 g, 2 mmol) with acryloyl chloride (0.34 mL, 2.6 mmol) in 20 mL dry THF by using of triethylamine as the catalyst. The resulting orange solid was purified by recrystallization from a 2:1 ethanol: chloroform solution mixture (83% yield).

2.4 Preparation of dual-color fluorescent nanoparticles

Typically and in an emulsion polymerization recipe, an aqueous solution of SDS/Triton X-100/$NaHCO_3$/KPS feed (0.06 g/0.03 g/0.03 g/0.03 g) was prepared, poured into a three-necked round bottom reactor, stirred and deaerated with nitrogen, respectively. Then the solution temperature reached to 70°C. Afterward, polymerization was started by adding monomers i.e. 60 mg SPEA in 5 mL DI water and a solution of 4.5 mg AzoCzEA in MMA (3.2 mL) from two separate dropping funnels simultaneously into the reactor within 20 min. The emulsion polymerization continued at 70 °C for 180 minutes until the conversion reached to above 95%. Finally, the resulting latex containing copolymer of AzoCzEA/SPEA/MMA was obtained and the coagulation was less than 1 wt%. More detailed information could be found elsewhere [15]. The nanoparticles were obtained by freeze-drying and then introduced into the dialysis bag (Mw cut-off: 12,400 Da). They were dialyzed against phosphate-buffered saline (PBS, pH=7.4, 1 L) which replaced every 12 h in the course of over 60 h to eliminate excess surfactants, unreacted monomers and short polymeric chains.

2.5 Delivery of DCPF Nanoparticles into DU145 living cells

Nanoparticles were delivered into DU145 cells by adding 1 mg/mL of the prepared dual-color fluorescent nanoparticle to each cell-containing well in the culture plate. After incubating for 24 hours at 37°C in a CO_2 incubator, the cells were washed with 500 µL PBS and fixed with 2% formaldehyde. The optical imaging was carried out using an inverted fluorescence microscope INV100-FL equipped with a CCD camera (BEL, Italia).

2.6 Cell viability assay

2×10^4 DU145 prostate cancer cells were seeded in 24-well plates (Nunc, Roskilde, Denmark). After 24 hours, the cells were treated with 0.1, 1, 5, 10, 15, 20 and 25 mg/mL of DCPF nanoparticles for

24 hours. After treatment, the single cells were tested for viability using Trypan blue dye exclusion assay. For this assay, a suspension of treated and control single cells from monolayer culture was mixed with Trypan blue at a 9:1 ratio. The resulting mixture was examined within 3–5 minutes under an optical microscope and blue-colored cells were considered dead. The ratio of unstained cells to the total number of cells was reported as the viability percentage for each cell category.

3 Result and discussion

Here, the donor and acceptor monomers based on azo-carbazole and spiropyran groups, respectively, were employed in preparation of DCPF polymer nanoparticles and used as a labeling agent by FRET phenomenon in the prostate cancer living cells. The chromophores were covalently incorporated into the PMMA matrix and identified thoroughly [15]. The energy transfer between SPEA and AzoCzEA and its kinetics, beside photoreversibility were investigated to reveal the efficiency of FRET for these polymer nanoparticles. Then they were dialyzed to prohibit any cytotoxicity. To approve the applicability of these nanoparticles, they were exposed to DU145 prostate cancer cells and their photoswitchable properties were examined by fluorescence imaging.

The conducted efficient copolymerization not only restricts undesirable prominent dye leakage and dye aggregation (**Figure 1**), but also enhances their photostability for long time, high contrast and brightness vastly. In addition, the consequence of these problems (arose from usual doping) is the decrease in FRET efficiency. To have reasonable energy transfer performance, the optimized ratio of chromophores must be kept unchanged with time. Beside essential efficient distance between these chromophores for obtaining best spectral overlap, dye leakage and aggregation will result in their segregation. These issues are responsible to suppress the FRET phenomenon in the doped particles.

To study photostability of the obtained nanoparticles, a latex dispersion was sealed and stored in dark at room temperature. Then, the diluted dispersion was utilized for fluorescence analysis after 12 weeks. The fluorescence intensity and photoswitching efficiency of the diluted latex did not change during these weeks, representing extensive long-term photostability and no dyes leakage for the prepared DCPF nanoparticles. These depict the improved fatigue resistance for such a system and this will become specifically important and compulsory for cell viability, while they are going to be employed in biomedical applications.

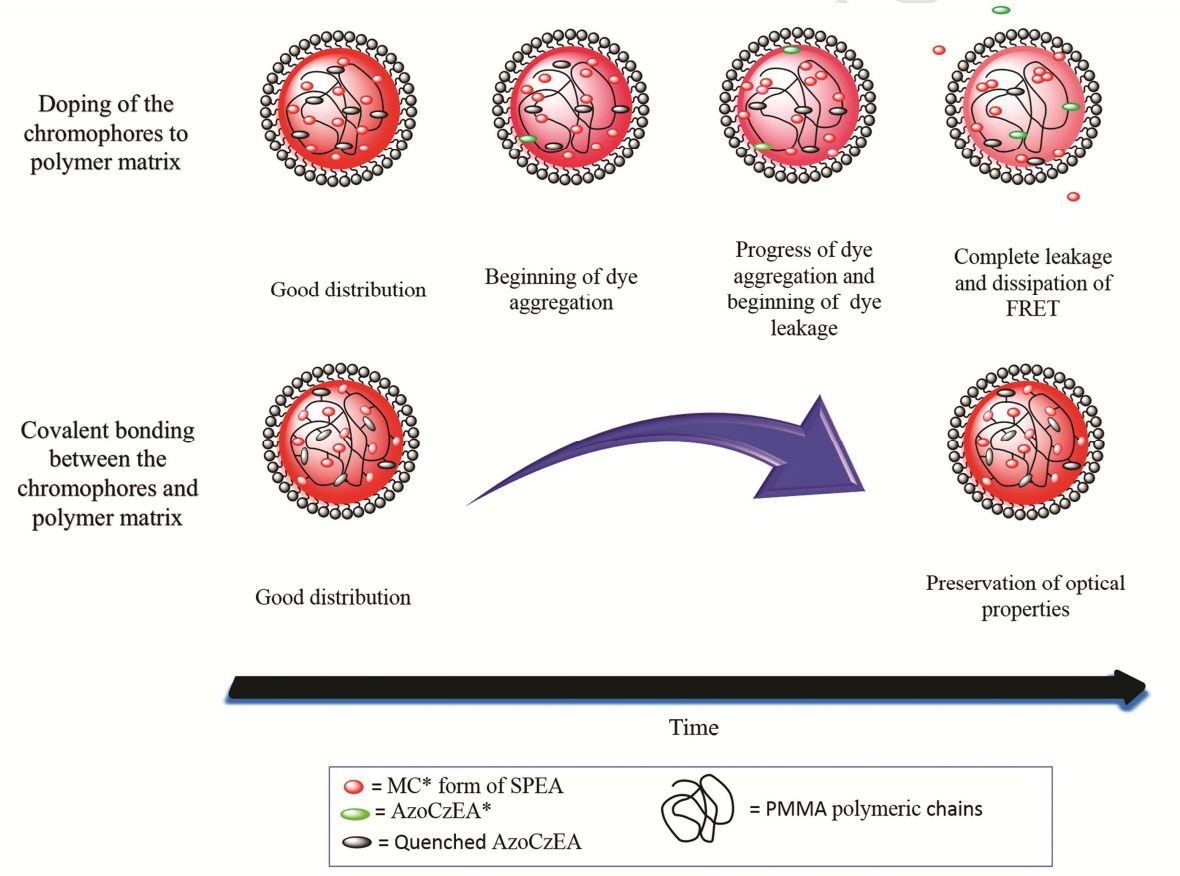

Figure 1. Schematic representation of dye leakage and aggregation with duration of time (objects are not to scale)

UV-Vis spectrum of DCPF nanoparticles containing AzoCzEA and SPEA showed an absorption band in 400-550 nm, which was attributed to the presence of AzoCzEA chromophores. When these stimuli-responsive nanoparticles are exposed to UV irradiation at 365 nm, a new absorption band at 500-650 nm appears that is the result of SP to MC isomerization. Disappearance of the absorption band at 430 nm with the exposure of nanoparticles by stimulating at 365 nm owes to the occurrence of fluorescence resonance energy transfer from the excited state of AzoCzEA to MC isomer of SPEA. Totally, by excitation of the above DCPF nanoparticles at 410 nm, they emit green fluorescence light and because of FRET after UV irradiation at 365 nm, this green fluorescence emission interchanges to red with respect to the excitation of MC isomer.

Variations in fluorescence intensity in 450-700 nm (green emission; λ_{max}=530 nm) with irradiation time at 365 nm have been represented in **Figure 2**.

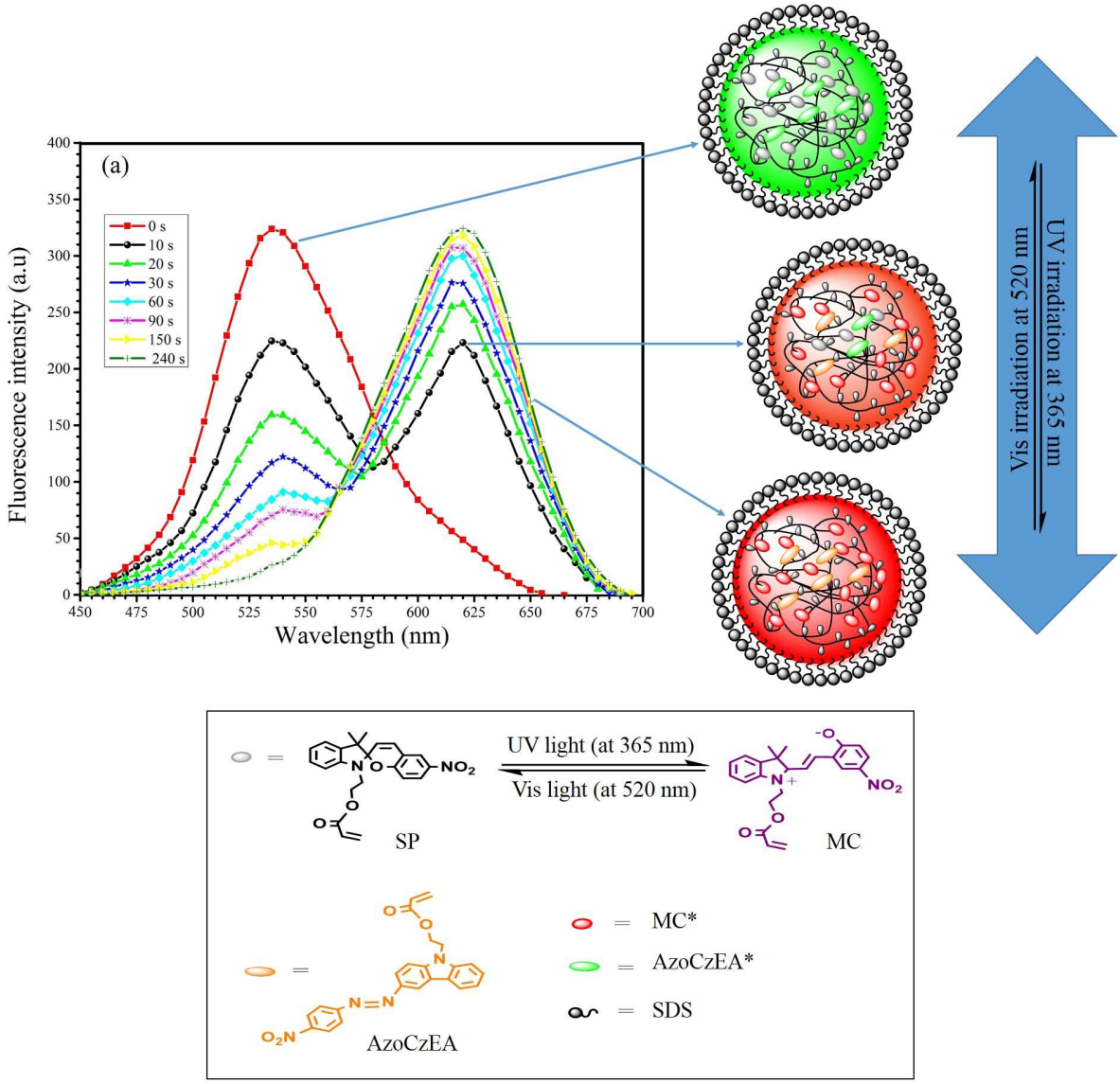

Figure 2. Time dependent fluorescence spectra of the prepared dispersion of DCPF nanoparticles in water upon UV irradiation at 365 nm

It is evident that the fluorescence intensity decreases with the increase in irradiation time and this returns to the increase in MC concentration at longer irradiation time at 365 nm that makes the quenching process proceed more. The green fluorescence intensity gradually decreases until reaches to zero after 240 s of UV irradiation at 365 nm. On the other hand, the red fluorescence emission (λ_{max}=620 nm) starts to appear, relating to the fluorescence emission of MC isomers.

This is a strong evidence for observing complete fluorescence quenching process (quenching efficiency above 98%, calculated by peak integration) in such DCPF nanoparticles as a consequence of efficient FRET phenomenon.

The recovery of green fluorescence emission could be achieved by the exposure of above particles to visible light (520 nm) upon transformation of MC to SP isomers. The complete recovery took about 7 min after visible irradiation in such systems and corresponding fluorescence intensities have been listed in **Table 1**.

Table 1. Fluorescence intensity (FI) under UV (365 nm) and visible (520 nm) irradiations at different time for DCPF nanoparticles*

Irradiation time (s)	0	10	20	30	60	90	120	150	190	240	300	360	420
FI after UV irradiation	322	221	160	119	88	70	-	47	-	22	-	-	-
FI after Vis irradiation	21	-	-	50	72	98	137	179	211	244	275	312	321

*Fluorescence intensities were recorded in 450-700 nm

The kinetics of fluorescence intensity of AzoCzEA moiety in DCPF nanoparticles and its recovery, according to the extracted data from **Table 1** and with respect to reversible SP to MC isomerization, could be fitted to the Equation 1 [26]:

$$\mathrm{Ln}\left((E_\infty - E_0)/(E_\infty - E_t)\right) = k_{iso}.t \qquad \text{Eq. 1}$$

Where E_0, E_t and E_∞ are the emission intensities (λ_{max}=530 nm) at time 0, t and infinity, respectively, and k_{iso} is the rate constant of SPEA isomerization.

Time profiles of emission for DCPF nanoparticles during UV/Vis irradiation exhibit a deviation from the first order kinetics. **Figure 3a** reveals that the fluorescence quenching carries out with a

constant rate (k_{iso} of 0.0222 s^{-1}). Facile energy transfer illustrates the accessibility of chromophores for immediate overlap between molecular orbitals. The duration and rate of energy transfer will depend on either mobility of the polymeric chains or the distance between MC isomer and AzoCzEA in DCPF nanoparticles to promote bringing them together. In other words, k_{iso} would be assumed as a parameter to indicate the vicinity of chromophores and mobility of polymeric matrix. The recovery of fluorescence emission occurs in slower rate primarily with k_{iso} of 0.0063 s^{-1} (**Figure 3b**). This process depends on the MC to SP isomerization rate entirely and is strongly controlled by the stabilization of MC zwitterions in the medium. The dipolar or ionic interactions of MC with the polymeric matrix (PMMA here) play important role for this issue. Consequently, bonded MC groups to the copolymer chains in DCPF nanoparticles and establishment of ionic and dipole-dipole interactions will reduce the mobility of polymeric chains. It has also been depicted that π-π stacking between aromatic structures improves the stability of MC isomers and retard isomerization rate of MC to SP [27]. This will cause in difficulty for keeping AzoCzEA away from tightly-fixed MC isomers and accordingly, slows down the rate of fluorescence recovery. The sudden increase in k_{iso} (0.0319 s^{-1}) after 300 s of visible irradiation is attributed to the significant decrease in MC concentration in DCPF nanoparticles and inaccessibility of residual MC isomers to AzoCzEA groups. This will result in remarkable regeneration of green fluorescence emission.

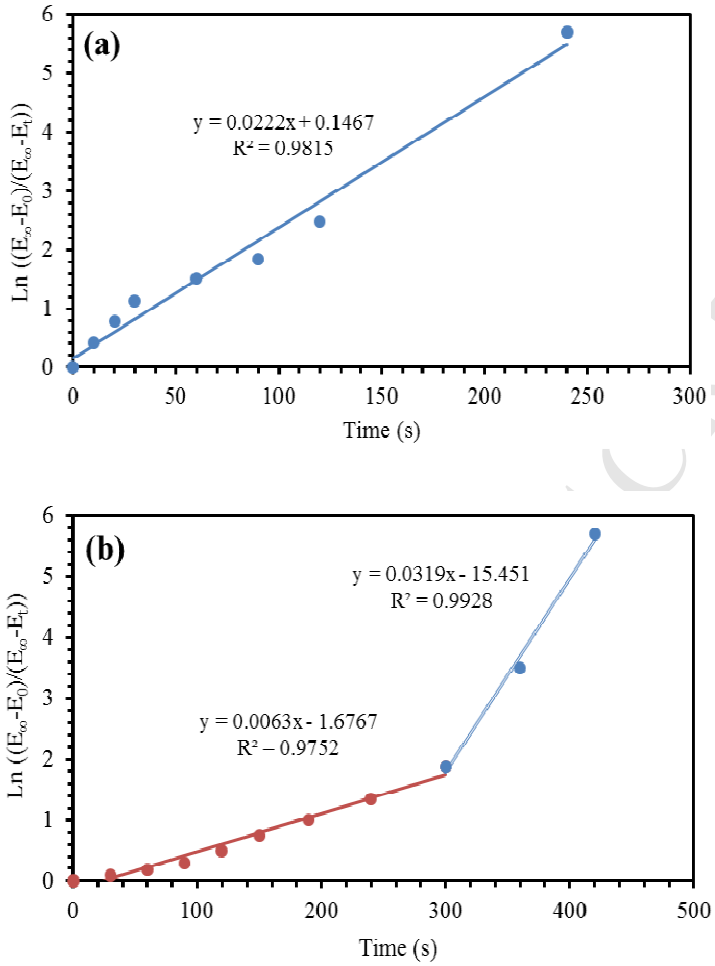

Figure 3. Fluorescence quenching of DCPF nanoparticles by UV (365 nm) (a) and its recovery by visible (520 nm) (b) irradiations with time

The effect of DCPF polymer nanoparticles on the viability of DU145 cells was determined by Trypan blue dye exclusion assay and shown in **Figure 4**. The cells were dealt with various amounts of DCPF nanoparticles and half maximal inhibitory concentration (IC50) for these fluorescent nanoparticles was calculated as 22 mg/mL.

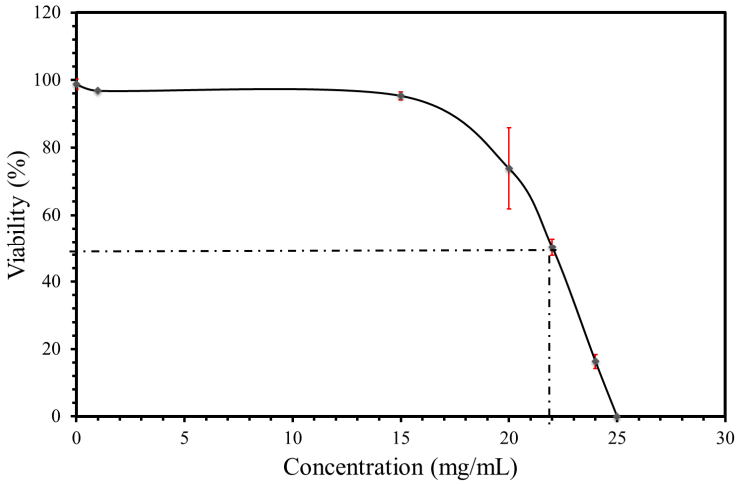

Figure 4. DU145 cells survival after treatment with different concentrations of DCPF nanoparticles for 24 h

This assay implies that below 22 mg/mL of the nanoparticles, they show still reasonable cell compatibility without significant toxification. Therefore, and with respect to the biocompatibility analysis, they could be exploited in biomedical devices without serious concern.

Therefore, imaging experiments were conducted to examine the potential application of these DCPF nanoparticles as bioimaging agents. Initial results indicated that the dual-color fluorescent nanoparticles were directly entered into DU145 living cells (**Figure 5**). It is suggested that these polymer nanoparticles are able to fuse to the prostate cancer cells membranes and internalize inside them without noticeable toxic effects on the live cells. DU145 cells including the dual-color nanoparticles show green fluorescence after excitation at 410 nm (**Figure 5**a), which is attributed to AzoCzEA, while SPEA moieties in DCPF has no fluorescence emission under this condition. The cellular imaging assays indicate that DCPF nanoparticles can be distinguished in the living cells explicitly. SP to MC isomerization under UV irradiation at 365 nm will lead to the formation MC isomer with fluorescing ability. According to the FRET phenomenon, MC can absorb energy from

the excited state of AzoCzEA after irradiation at 410 nm. The excited MC moieties will consequently fluoresce in red at 630 nm. Thus, the green fluorescence is quenched after irradiation at 365 nm and the red fluorescence will be observed (**Figure 5b**). **Figure 5c** represents the bright field image of **Figure 5a**. It could be observed that the green fluorescence of DCPF polymer nanoparticles is detectable in bright field (arrows) due to the excitation at 410 nm. These smart fluorescent probes demonstrate promising potentiality in selectively highlighting systems such as cell labeling, cell imaging and photo-tracking with high contrast fluorescence. It would be of great importance for having the capability to discriminate labelled cell with DCPF nanoparticles from autofluorescence and fake signals of the background.

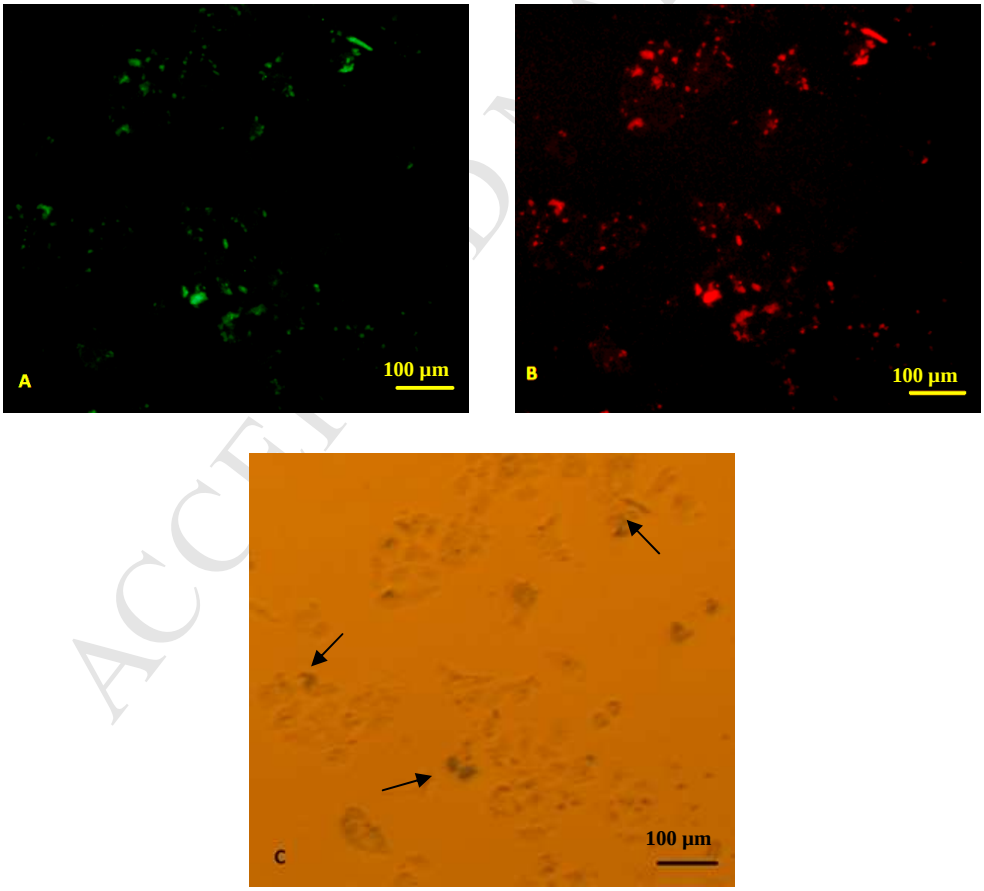

Figure 5. Fluorescence microscopy images of the prepared dual-color photoswitchable fluorescent nanoparticles in DU 145 human prostate cancer cells after excitation at 410 nm (a) before and (b) after UV irradiation at 365 nm. (c) is the bright field image of (a) (magnifications are 10X).

In this study, photoresponsive fluorescent acrylic polymer nanoparticles were employed in phototracking and labeling of human prostate cancer cells by UV stimulus. Results of fluorimetry analyses reveal that the prepared DCPF nanoparticles have prominent photostability and photoreversibility that make them suitable for biomedical applications. Kinetic evaluation for the FRET phenomenon, fluorescence quenching and its recovery under UV/Vis irradiation were investigated extensively. The obtained results were attributed to polymer chain mobility, π-π stacking between aromatic structure and interaction of chromophores with polymer chains. Covalent bonding between chromophores, as a great advantage, makes excellent distribution of the chromophores in polymer chains and preservation of FRET and also enhanced photoreversibility and fatigue resistance in such DCPF nanoparticles. The obtained dual-color latex was purified by dialysis and a dispersion of DCPF fluorescent nanoparticles were delivered into DU145 cancer cells. Cell viability assays showed good biocompatibility with reasonable toxicity for DCPF nanoparticles. Results of fluorescence microscopy images indicated excellent cell uptake and dual-color fluorescence of the labeled prostate cancer cells. It is believed that the introduced photoswitchable fluorescent polymer nanoparticles can be used as a promising candidate for cell labeling and tracking to remove the probable fake background signals from original ones in precise detections.

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FRET-based acrylic nanoparticles with dual-color photoswitchable properties in DU145 human prostate cancer cell line labeling

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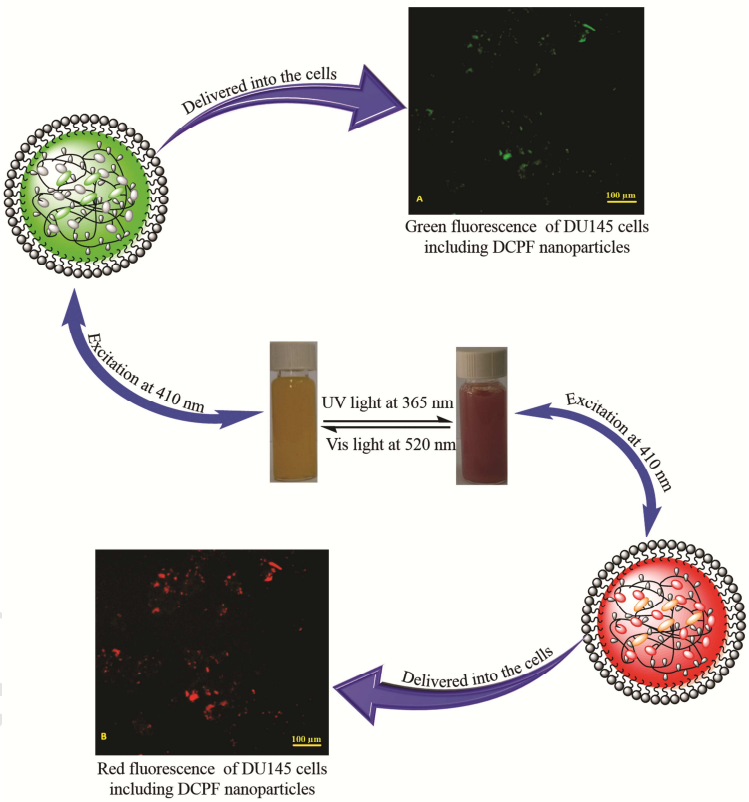

- Preparation of photoswitchable fluorescent polymer nanoparticles as cell detector
- Dual-color acrylic nanoparticles containing spiropyran and azo-carbazole derivatives
- FRET- based particles with improved photostability and photoreversibility
- Reasonable cell compatibility and cell uptake to prostate cancer cells