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LETTER

# Tunable and Selective Detection of Cancer Cells using Betainized Zwitterionic Polymer with BODIPY and Graphene Oxide

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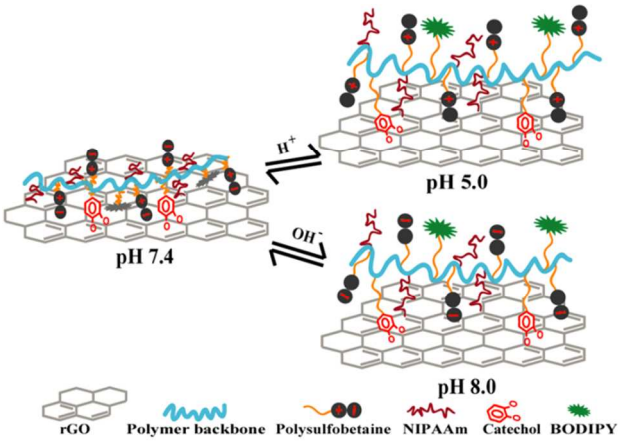
**A novel type of zwitterionic fluorescent nanoparticles (ZFNPs) containing polysulfobetaine groups with boron dipyrromethane (BODIPY) fluoro-phores and graphene oxide plates is prepared for the detection of tumor cells in response to the intra- and extracellular stimuli. The fluorescence intensities of the ZFNPs are highly influenced by temperature and pH.**

Multifunctional nanoparticles are widely used as imaging probes, biosensors, and targeted drug delivery.[1,2] These efforts have been driven to a great extent by the need to improve biological specificity in diagnosis and therapy.[3,4] As a result, continuous efforts have been dedicated to the development of stimuli-responsive nanoplatforms. Environmental stimuli that were exploited include temperature, pH, enzymatic expression, redox reaction, salt concentration, and light all be used to study of biological molecules, pathways, and processes in living cells.[4-6] Among these activating signal, pH triggers the most extensively studied stimuli based on the differences of pH between pathological (e.g. tumor) versus normal tissues and acidic intracellular compartments.[7,8] To target the acidic endosomal or lysosomal compartments, several smart nanoplatforms with pH-induced charge conversion were designed to improve cellular imaging and drug efficacy.[9] For instance, graphene oxide (GO), an atomic thin carbon material, is widely used in biosensing, cell imaging and drug delivery combining with pH-sensitive fluorescence dye molecules.[10] The unique capacity of GO in adsorbing polymeric molecules on its surface with super fluorescence quenching efficiency creates a robust platform for the development of fluorescence probes.[10,11]

Until recently, the physical characteristics of nanoparticles such as size, shape, hydrophobicity, and net charge have been mostly considered as primary handles to achieve nanoparticles with desired cell imaging characteristic.[3,4,6] Recently, zwitterionic polymers such as polysulfobetaine, polycarboxybetaine and polyphosphorylcholine have been widely used as a new gene delivery vector, and to resist non-specific protein adsorption and cell adhesion for surface treatment to afford antibiofouling properties whereas these polymers exhibit strong stimuli-responsiveness such as pH, temperature, and salt concentration.[12-15]




Scheme 1. Scheme illustrate the plausible mechanism for fluorescence behaviors of **(CA-BODIPY)-PSMN**/rGO with different pH in phosphate buffer solutions at physiological temperature.

Herein, we designed a specific type of zwitterionic fluorescent nanoparticles (ZFNPs), poly (sulfobetaine methacrylate-co-N-isopropylacrylamide-g-catechol-BODIPY)/rGO [**(CA-BODIPY)-PSMN**/rGO], for investigating pH and temperature responsive

fluorescence properties. To develop the ZFNPs, we primarily employ random copolymer poly[2-(dimethylamino) ethyl methacrylate-co-N-isopropylacrylamide] [poly (DMA-co-NIPAAm), (PDN)] using radical polymerization according to published literature.[16,17] Then, a robust catechol molecule 2-chloro-3', 4'-dihydroxyacetophenone (CA) and fluorescent dye molecule boron-dipyrromethane with benzyl chloride (BODIPY) were conjugated to DMA to form [(CA-BODIPY)-PDN].[18] After that, the betainization of the copolymer (CA-BODIPY)-PDN was carried out in a co-solvent THF/ethanol (1:1) by using 1.3-propane sultone to get **(CA-BODIPY)-PSMN** (Fig. S1). Finally, a highly efficient fluorescence quencher GO was chemically reduced using mussel inspired catechol moiety to form ZFNPs **(CA-BODIPY)-PSMN**/rGO.[8]

We hypothesized that fluorescence characteristic of the prepared NPs depends on pH and temperature where zwitterionic polysulfobetaine and NIPAAm play the vital role through interaction with GO sheets. Scheme 1 illustrates the plausible mechanism of the proposed ZFNPs. In the acidic environment, highly hydrophilic nature of the cationic ZFNPs through protonation of sulfobetaine group result in strong green fluorescence of BODIPY dye. The measured zeta potential at acidic environment of pH 5.0 was +22.4 mV and at pH 6.0 was +14.5 mV indicating the cationic nature of the zwitterionic **(CA-BODIPY)-PSMN**/rGO (data not given). However, at neutral pH values, the fluorescence intensity were totally quenched due to the interaction of neutral **(CA-BODIPY)-PSMN** polymer and GO surfaces. At this condition, the values of zeta potential was decreased to -0.8 which proved almost nonionized form of the ZFNPs. Furthermore, in the basic environment, the fluorescence intensity was recovered due to deprotonation of the sulfobetaine groups that makes anionic form of zwitterionic **(CA-BODIPY)-PSMN**/rGO and increases the hydrophilicity (zeta potential value -5.7 mV).

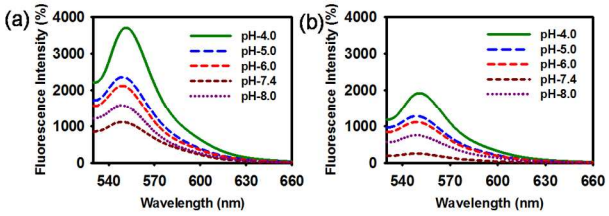



**Fig. 1** (a) and (b) fluorescence spectra of **(CA-BODIPY)-PSMN**/rGO (0.01mg/ml) at different pH with room and body temperature in phosphate buffer solutions. The excitation wavelength is 526 nm.

The fluorescence intensities of **(CA-BODIPY)-PSMN**/rGO was measured in phosphate buffer solutions (PBS) by altering temperature and pH. Fluorescence intensities of **(CA-BODIPY)-PSMN**/rGO were mostly affected by changing temperature and pH (Fig. 1). At lower pH, very high fluorescence intensities were observed in both room and body temperature. However, the fluorescence intensities were almost quenched at pH 7.4 at body temperature and no detectable fluorescence was found at this condition (Fig. 1b). The quenching mechanism can be attributable to the formation of neutral betainized **(CA-BODIPY)-PSMN** that can interact non-covalently with rGO via collisional and static quenching mechanism.[8,18] Furthermore, the fluorescence intensities were recovered in basic environment in both room and physiologic temperatures (Fig. 1b). This phenomenon can be rationalized by the hydrophilicity of the **(CA-BODIPY)-PSMN**/rGO via deprotonation of the zwitterionic sulfobetaine group in the polymer backbone. We also measured the fluorescence intensities of **(CA-BODIPY)-PSMN** without GO at different pH with room and body temperature (Fig. S2). Such a fluorescence enhancement might be attributable to the absence of superquencher GO surfaces. Although the fluorescence intensities decreased at neutral pH and body temperature, they were not fully quenched. This result indicates that the GO plate has an important role to play in quenching the fluorescent nanoparticles.[8]

Atomic force microscope (AFM) analysis was performed to obtain the photographical profile of the synthesized **(CA-BODIPY)-PSMN**/rGO fluorescent nanoparticles by altering the pH condition. Interestingly, different heights were observed in results of changing pH. **(CA-BODIPY)-PSMN**/rGO plates ranging from 100 nm to 130 nm in lateral dimension were observed in AFM analyses and the average height at pH 7.4 was 7.3 nm (Fig. 2b) which confirming the neutral zwitterionic polysulfobetaine deposition onto the surface of the GO. The average thickness in acidic (pH 5.0, 4.2 nm) and basic (pH 8.0, 5.8 nm) condition was lower than normal tissue pH (Fig. 2a and 2c) which was attributable to the protonation and deprotonation of the sulfobetaine group resulting in more hydrophilic polymer backbone with decreasing the depth of **(CA-BODIPY)-PSMN**/rGO. The particle size of the **(CA-BODIPY)-PSMN**/rGO ZFNPs was also quantified by using dynamic light scattering (DLS) at different pH (Fig. S3). All particles in different pH exhibited hydrodynamic sizes ranging from 120-160 nm, which are in good agreement with AFM results. Therefore, the combination of AFM and DLS results confirmed that the **(CA-BODIPY)-PSMN**/rGO ZFNPs are highly responsive to pH and can be obtained on the nanoscale without apparent aggregation suggested high aqueous stability. The elemental composition of **(CA-BODIPY)-PSMN**/rGO was also confirmed via X-ray photoelectron spectroscopy (XPS). The survey scan spectrum of **(CA-BODIPY)-PSMN**/rGO confirmed the main peaks of C 1s and O 1s centered at 284 and 533 eV, respectively (Fig. S4a). Core-line spectrum of nitrogen revealed two characteristic peaks of neutral amine and cationic amine at 399.0 and 401.8 eV that confirmed the quaternization of CA and BODIPY (Fig. S4c).

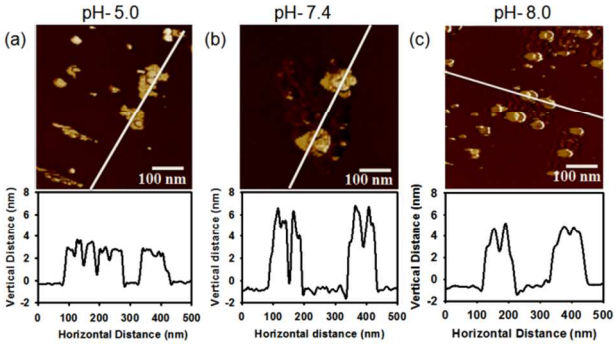



**Fig. 2** Atomic force microscope (AFM) images and height profile of a droplet of **(CA-BODIPY)-PSMN**/rGO at different pH conditions (a) pH 5.0, (b) pH 7.4, and (c) pH 8.0 in phosphate buffer solutions respectively.

To investigate the intracellular activation of ZFNPs, we examined **(CA-BODIPY)-PSMN**/rGO in human MDA-MB 231 breast cancer cells by confocal laser scanning microscopy (CLSM) at pH between 5.0 and 8.0 in Fig. 3a. It should be noted that **(CA-BODIPY)-PSMN**/rGO ZFNPs have an optimal pH changes at around 5.0 to 6.0, which are ideally suited to the nanoparticle activation in endosomes and lysosomes. Because **(CA-BODIPY)-PSMN**/rGO ZFNPs are silent at neutral pH values, we directly applied them in the culture medium and monitored the kinetics of their uptake and activation without the need to remove the medium. In this environment, neither the MDA-MB 231 cells nor the medium showed an observable fluorescence signal. To get the endosomal pH, we added 0.1 N HCl solutions in the cell medium. Fascinatingly, considerable increases in fluorescence intensity in the medium background and inside the cell cytoplasm were found. Furthermore, by adding 0.1 N NaOH solutions to the cell medium to make basic environment, the fluorescence intensity was recovered and consequently, green fluorescence was observed at pH 8.0. All these results indicate that the synthesized **(CA-BODIPY)-PSMN**/rGO ZFNPs are highly influenced by pH and could be allowed the selective detection of cancer cells. Fig. 3b shows the location of lysosomes stained by LysoTracker Red. It was found that the Red emission of Lysotracker and green emission of **(CA-BODIPY)-PSMN**/rGO co-localize and overlay very well with each other in the live cells which assured the localization of ZFNPs in lysosomes (Fig. 3b). However, the fluorescence emission was almost quenched at physiological pH for both **(CA-BODIPY)-PSMN**/rGO and LysoTracker which further proved the pH sensitivity of the ZFNPs.

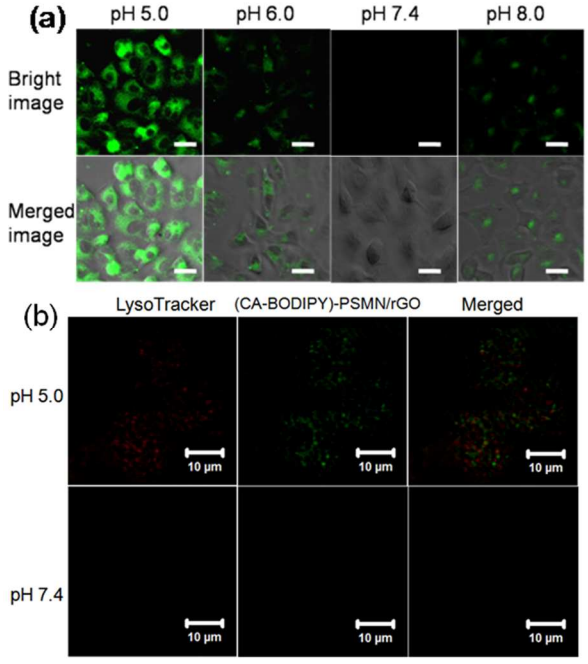



**Fig. 3** (a) Confocal microscope image of human MDA-MB 231 breast cancer cells after incubation with **(CA-BODIPY)-PSMN**/rGO ZFNPs in fresh culture medium at different pH values. All the scale bars are 20 μm. (b) Subcellular localization of **(CA-BODIPY)-PSMN**/rGO in MDA-MB 231 cells incubated with LysoTracker and **(CA-BODIPY)-PSMN**/rGO and overlap.

Confocal microscopy investigations were determined at the fluorescence properties of **(CA-BDP)-PSMN**/rGO incubated in Hela cancer cells and MDCK normal cells at pHs between 5.0 and 8.0 (Fig. 5S). It is noted that the fluorescence intensities gradually increased under the acidic and base conditions and consequently fluorescence signals were not observed at pH 7.4. These results indicate that the **(CA-BDP)-PSMN**/rGO could be used on/off switching properties for the detection and monitoring of cancer cells. The cytotoxicities of **(CA-BODIPY)-PSMN** and **(CA-BODI-PY)-PSMN**/rGO were assessed by [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (MTT) assays after incubation with MDA-MB 231 cells for 24 h (Fig. S6a). The **(CA-BODIPY)-PSMN** polymer and **(CA-BODIPY)-PSMN**/rGO ZFNPs have no visible cytotoxic effect on MDA-MB 231 cells after 24 h treatment, even at concentration of 0.5 mg/ml indicating that **(CA-BODIPY)-PSMN**/rGO ZFNPs possess excellent biocompatibility which is attributable to the zwitterionic polysulfobetaine block expected to act as a beneficial element to lower the toxicity of GO nanocarrier.[19] To further elucidate the cellular damage caused by **(CA-BODIPY)-PSMN**/rGO nano-particles, a LIVE/DEAD cell assay was performed by using MDA-MB 231 cell line. The cells were imaged by confocal microscopy with a dual-dye system where calcein AM (green) permeates intact live cells and membrane-impermeable dye propidium iodide (red) was used to identify the dead cells. Untreated cells showed completely green fluorescence indicating live cells (Fig. S6b). Moreover, MDA-MB 231 cells show high green fluorescence and a very little amount of red fluorescence after incubated with 0.5 mg/ml of both **(CA-BODIPY)-PSMN** and **(CA-BODIPY)-PSMN**/rGO, indicating the considerable cell viability.

In conclusion, we have successfully developed a novel GO-based zwitterionic fluorescent nanoparticle for highly sensitive and selective detection of cancer cells via imaging and this platform can be expanded to drug delivery systems (DDS) through imaging and treatment of cancer cells through loading of anticancer therapeutics. The fluorescent NPs showed strong fluorescent at acidic and basic condition and almost non fluorescence at physiological pH and temperature. Confocal microscopic image also showed intense fluorescence at endosomal pH values but no fluorescence signal under normal body pH conditions. The unique fluorescence emission property and facile fabrication methods of zwitterionic **(CA-BODIPY)-PSMN** to GO sheets provide great potential of these ZFNPs to be applied for molecular diagnostic as a novel fluorescent probe.

## Experimental

### Synthesis of catechol conjugated poly (DMA-co-NIPAAm) (CA-PDN)

To prepare CA-PDN via quaternization, PDN (0.1 mM) and 2-chloro-3', 4'-dihydroxyacetophenone (CA, 2 mM) were dissolved in 50 ml of ethanol in a 250 ml flask. The mixture was stirred for 48 h at 70 $^0$C. After stirring, the solvent was evaporated in a rotary evaporator and precipitated using cold diethyl ether. The resulting CA-PDN was dried in vacuum. The yield of the product was 77%.

$^1$H NMR (400 MHz, $D_2O$, δ): 0.4-0.9 (3H, *$CH_3$*), 1.8-2.25 (6H, (*$CH_3$*)$_2$ of DMA), 3.95 (1H, -*CH* ($CH_3$)$_2$ of NIPAAm), 6.69 (1H, *CH* of catechol), 7.32 (2H, *CH* of catechol). *Mn* = 38,800.

**Conjugation of BODIPY to CA-PDN [(CA-BODIPY)-PDN]**

The process of BODIPY conjugation was similar to CA conjugation with CA-PDN. For a typical reaction, CA-PDN (0.03 mM) and BODIPY with benzyl chloride (0.1 mM) were dissolved in 30 ml of ethanol and stirred 48 h at 70 $^0$C.[20] Following reaction time, the solvent was evaporated using rotary and precipitated in cold diethyl ether. The product was then dried in vacuum and analyzed. The yield of the product was 83%.

$^1$H NMR (400 MHz, $CDCl_3$, δ): 0.94 (6H, $CH_3CH_2$- of BODIPY), 1.28 (6H, -$CH_3$ of BODIPY), 2.0-2.25 (6H, N($CH_3$)$_2$ of DMA), 2.29 (4H, $CH_3CH_2$- of BODIPY), 4.0 (1H, -$CH$ ($CH_3$)$_2$ of NIPAAm), 7.28 (2H, aromatic protons), 7.53 (2H, aromatic protons). *Mn* = 39,600

**Synthesis of poly (sulfobetaine methacrylate-co-NIPAAm conjugated catechol and BODIPY) [(CA-BODIPY)-PSMN] through betainization**

The betainization of poly (DMA-co-NIPAAm) conjugated catechol and BODIPY) (CA-BODIPY)-PDN (0.02 mM) was achieved simply by adding 1, 3-propanesultone (3 mM) (10 mol% excess based on DMA residues) in co-solvent THF/ethanol with 1 to 1 ratio at room temperature. The solution was then stirred for 24 h at 40$^o$C. The resulting **(CA-BODIPY)-PSMN** was purified via precipitation into n-hexane after rotary evaporation. The product was then dried in vacuum at room temperature for at least 2 days. The extent of betainization was assessed by $^1$H-NMR spectroscopy. The yield of the product was 74%.

$^1$H NMR (400 MHz, $D_2O$, δ): 1.12-1.21 (6H, ($CH_3$)$_2$ of NIPAAm, 3.78 (1H, -$CH$ ($CH_3$)$_2$ of NIPAAm), 3.14-3.46 (6H, ($CH_3$)$_2$ of DMA, 2.88 (2H, -$CH_2$-$SO_3$), 2.42 (2H, -$CH_2$-$CH_2$-$SO_3$), 3.63 (2H, -$CH_2$-$CH_2$-$CH_2$-$SO_3$), 6.95 (2H, aromatic protons), 7.45 (2H, aromatic protons). *Mn* = 41,500.

**Preparation of Reduced Graphene Oxide [(CA-BODIPY)-PSMN/rGO]**

Graphene oxide (GO) was synthesized by the modified Hummers method using nature graphite oxide.[21] To prepare reduced graphene oxide, 200 mg **(CA-BODIPY)-PSMN** in 8 ml 10 mM tris buffer at pH 8.5 was mixed with 2 ml (1mg/ml) aqueous suspension of graphene oxide (GO) platelets. The pH of the solution was adjusted to 8.5 and stirred for 24 h at 40 $^0$C. Finally, the solution was purified following dialysis (molecular weight cut-off: 3,500) and freeze dried to get the **(CA-BODIPY)-PSMN/**rGO powder.

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## Notes and references

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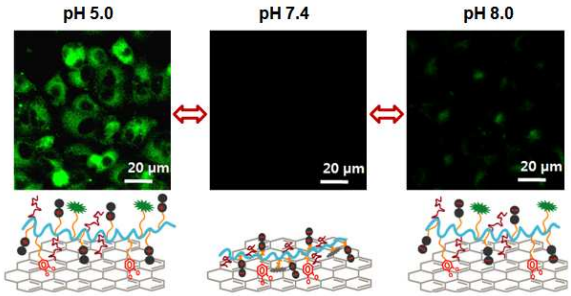


**Novel fluorescence probes, reduced graphene oxide (rGO) containing Zwitterionic fluorescence nanoparticles, for effective diagnosis of cancer cell.**