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RESEARCH ARTICLE

BODIPY-conjugated chitosan nanoparticles as a fluorescent probeGizem Bor¹, Muhammed Üçüncü², Mustafa Emrulloğlu², Aysel Tomak³, and Gülşah Şanlı-Mohamed²¹Biotechnology and Bioengineering Department, ²Chemistry Department and ³Materials Science and Engineering Department, Izmir Institute of Technology, Izmir, Turkey**Abstract**

Recently, development of fluorescent nanoparticle-based probes for various bioimaging applications has attracted great attention. This work aims to develop a new type fluorescent nanoparticle conjugate and evaluate its cytotoxic effects on A549 and BEAS 2B cell lines. Throughout the study, ionically crosslinked chitosan nanoparticles (CNs) were conjugated with carboxylated 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY-COOH). The results of conjugates (BODIPY-CN) were investigated with regard to their physico-chemical, optical, cytotoxic properties and cellular internalization. The morphology of BODIPY-CN was found to be spherical in shape and quite uniform having average diameter of 70.25 ± 11.99 nm. Cytotoxicity studies indicated that although BODIPY-COOH itself was quite toxic on both A549- and BEAS 2B-treated cells, CNs increased the cell viability of both cell lines via conjugation to BODIPY-COOH fluorescent molecule up to 67% for A549 and 74% for BEAS 2B cells. These results may suggest a possible utilization of the new fluorescent nanoparticle-based probe for bioimaging in biology and medicine.

Keywords

Chitosan, nanoparticles, BODIPY, cytotoxicity, A549 cells, BEAS 2B cells

HistoryReceived 22 March 2016
Revised 11 August 2016
Accepted 9 September 2016**Introduction**

In recent years, bioimaging applications with fluorescent probes have attracted important attention in biology and medicine (Chekina et al., 2011; Corr et al., 2008; Terai & Nagano, 2008; Yuan et al., 2013). Especially, bioimaging applications with fluorescent molecules on cancer cells are highly promising with the possibility of real-time visual detection of an analyte both in solution and living organism (Chan et al., 2012).

Organic fluorescent dyes have been greatly applied in the synthesis of fluorescent probes for molecular imaging and biomedical diagnosis (Kobayashi et al., 2010). Boron-dipyrromethene (BODIPY), a type of organic fluorescent molecules, has remarkable photophysical properties such as long excitation/emission wavelengths, high molar absorption coefficients and fluorescence quantum yield (Boens et al., 2012; Ulrich et al., 2008; Loudet & Burgess, 2007). Thus, the BODIPY fluorophore can be a good candidate to be utilized as the organic fluorescent molecules for bioimaging applications (Bernhard et al., 2010). On the other hand, organic dyes are not very stable due to photobleaching problem, low-signal intensities and wide fluorescent spectrum limitations. It has lately been reported that, conjugation of organic dyes with polymer-based nanoparticles has increased their stability

compared to common single organic fluorophores (Nune et al., 2009).

Chitosan nanoparticles have attracted a remarkable attention lately. As a potential biomedical material, chitosan, a poly-N-acetyl glucosamine, is the N-deacetylated product of chitin (major component of crustacean shells), which is the second most abundant biopolymer after cellulose (Kumar et al., 2004). Chitosan, a type of cationic polysaccharide, has many significant biological and chemical properties due to a unique set of characteristics that include biocompatibility, biodegradability, nontoxicity, physiological inertness and antibacterial properties (Rinaudo, 2006). Therefore, chitosan can be used for nano-sized materials in biomedicine for variety applications (Jayakumar et al., 2010). Chitosan as nanoparticles are reported to be a suitable polymer-based material for loading organic fluorophores because of their high labeling affinity to various biomolecules (Zhao & Wu, 2006).

Although, many studies have been conducted for the development of chitosan nanoparticles with variety of fluorescent probes for the purpose of bioimaging applications, studies of BODIPY conjugated chitosan nanoparticles have not been reported. The objective of this study is to obtain BODIPY-conjugated chitosan nanoparticles as fluorescent probe and evaluate their cytotoxic effect on A549 and BEAS 2B cell lines. In the development of a functional fluorescent nano-size probe, initially, CNs were prepared based on ionic gelation method and BODIPY-COOH fluorescent dye was synthesized as a type of organic fluorescent molecule. CNs were then conjugated with BODIPY-COOH and the

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conjugation was confirmed by visualizing the newly formed compound under UV light and confocal microscope. The physicochemical properties of the CNs and BODIPY-COOH conjugated CNs were determined by several methods including Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FT-IR), Dynamic Light Scattering (DLS), Atomic Force Microscopy (AFM). Cytotoxic effects of CNs, BODIPY-COOH and BODIPY-CNs were evaluated comparing cell viability of A549 and BEAS 2B cell lines. According to our results, a newly formed fluorescent nanoparticle-based probe could be a good candidate for potential bioimaging applications in biology and medicine.

Materials and methods

Materials

Chitosan (190–310 kDa and degree of acetylation 75–85%), tripolyphosphate sodium (TPP), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylterazolium bromide (MTT) and 4',6-diamidino-2-phenylindole (DAPI) were obtained from Sigma-Aldrich (St. Louis, MO). All the other chemicals were obtained from local chemical suppliers.

Synthesis of BODIPY-COOH

Carboxylated 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY-COOH) was synthesized by the method of Brizet et al. (2013) with some modification. In the procedure, to an anhydrous dichloromethane (DCM) (50 mL) of 2,4-dimethylpyrrole (DMAP) (739.2 mg, 6.0 mmol), 4-carboxy-benzaldehyde (450.4 mg, 3.0 mmol) and catalytic amount of trifluoroacetic acid (2–3 drops) were added. The reaction mixture was stirred at room temperature for 24 h. A solution of p-chloranil (1.475 g, 6.0 mmol) in dichloromethane (10 mL) was added and the mixture was stirred for 1 h. Triethylamine (6 mL, 45 mmol) and $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (6 mL, 47.4 mmol) were added successively. The purple solution was stirred 4 h. The reaction mixture was extracted with dichloromethane (3×100 mL), dried over sodium sulfate and concentrated under vacuum. The residue was purified by silica gel column chromatography (Hex:EtAc 4:1). The resulting solid was further purified by recrystallization (chloroform-hexane system) to give title product as a red solid (Yield: 35%, $m = 445$ mg). ^1H NMR (400 MHz, CDCl_3): δ 8.26 (d, $J = 8.4$ Hz, 2H), 7.46 (d, $J = 7.4$ Hz, 2H), 2.54 (s, 6H), 2.31 (q, $J = 7.6$ Hz, 4H), 1.28 (s, 6H), 0.99 (t, $J = 7.6$ Hz, 6H). ^{13}C NMR (100 MHz, CDCl_3): δ 171.0, 154.4, 141.7, 138.4, 138.0, 133.1, 130.8, 130.2, 129.7, 128.9, 17.1, 14.6, 12.6, 11.8.

Preparation of chitosan nanoparticles (CNs)

Chitosan nanoparticles (CNs) were synthesized by following the Calvo's ionic gelation procedure (Calvo et al., 1997). According to this procedure, tripolyphosphate (TPP) was used as a crosslinker with chitosan. Chitosan was dissolved in 1% (w/v) acetic acid solution. Then, the pH of the solution was adjusted to 5.0 with 3.0 M NaOH solution. The aqueous solution of TPP was obtained as 0.1% in acetic acid solution. Chitosan nanoparticles started to form spontaneously upon addition of

TPP into the solution of chitosan under magnetic stirring drop wise at room temperature. After one hour of mixing chitosan with TPP, the solution was centrifuged (13500 rpm/30 min) and the pellet was freeze-dried by lyophilization.

Preparation and characterization of BODIPY-CNs

For preparation of BODIPY-CNs, freeze-dried chitosan nanoparticles were added to 5 mL of DMSO under constant magnetic stirrer. To this solution, BODIPY-COOH solution [5 mg in 1 mL DMSO and preactivated with 0.5 mg EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide reagent in 1 mL of pH 5.0 NaP buffer] was added drop wise. The reaction between CNs and BODIPY-COOH was allowed to continue for 24 h on the magnetic stirrer at room temperature. BODIPY-CNs were then centrifuged at 13 500 rpm for 30 min and washed with DMSO several times until the free BODIPY-COOH was not left in the supernatant. The BODIPY-COOH concentration was measured by UV-visible Spectrophotometry (Shimadzu-UV 2540, Kyoto, Japan) calibrated with standard solutions containing 0.005–0.5 $\mu\text{g}/\text{mL}$ of BODIPY-COOH dissolved in DMSO. Binding efficiency (w/w %) of the conjugate was calculated at the maximum wavelength of 510 nm.

The measurement of particle size (Z-average) of nanoparticles were performed on a Zetasizer Nano-ZS (Malvern Zetasizer, 3000 HSA, Worcestershire, UK) on the basis of dynamic light scattering (DLS) techniques. The measurements were performed in triplicates at a temperature of 25 °C and the samples were diluted with distilled water and measured at scattering angle of 173°. Changes in functional groups of chitosan nanoparticle after being conjugated with BODIPY-COOH were characterized by FT-IR (Perkin Elmer Spectrum 100, Waltham, MA) according to the potassium bromide pellet method on a Tensor-27 (Bruker, Billerica, MA) in the range of 800–4000 cm^{-1} . Visualization of the morphology of the particles was performed by Atomic Force Microscope on tapping mode (Digital Instruments Nanoscope, Santa Barbara, CA) and Scanning Electron Microscope (SEM, FEI QUANTA 250 FEG, Hillsboro, OR).

The optical property of BODIPY-CNPs was determined by recording the fluorescence emission spectra using excitation at the range of 400–500 nm with a fluorescence spectrophotometer (Perkin Elmer).

Cell culture

BEAS 2B (human bronchial epithelial cell line) and A549 (adenocarcinoma human alveolar basal epithelial cells) cell lines were provided by the Biotechnology and Bioengineering Research and Application Center, IZTECH. The cells were grown in Roswell Park Memorial Institute-1640 (RPMI-1640) growth medium containing 10% fetal bovine serum (FBS) and 1% gentamicin sulfate in a humidified atmosphere containing 5% CO_2 at 37 °C. Typically, cells were passaged through trypsinization and in growth medium.

In vitro cellular uptake of BODIPY-CNs

For the visualization of cells treated with BODIPY-CNs and BODIPY-COOH, confocal microscopy (Olympus-CKX41,

Tokyo, Japan) was used for A549 cell lines. The cells of passage 14 were seeded on Lab-Tek[®] chambered cover glasses (Thermo Scientific[™], Waltham, MA) at a density of 1×10^4 cells/cm² and incubated overnight at 37 °C, 5% CO₂ and 95% relative humidity. Then, the cells were treated with 0.2 mg/mL BODIPY-COOH and BODIPY-CN_s in 20 μL of transport medium for 4 h. The cells were washed twice with

phosphate-buffered saline (PBS), fixed in 3.7% paraformaldehyde and stained with DAPI (4',6-diamidino-2-phenylindole) before imaging. The applied excitation and emission wavelengths of DAPI dye in this study were 350 and 470 nm, respectively. The excitation and emission wavelengths used here for BODIPY-COOH and BODIPY-CN_s were 532 and 640 nm, respectively.

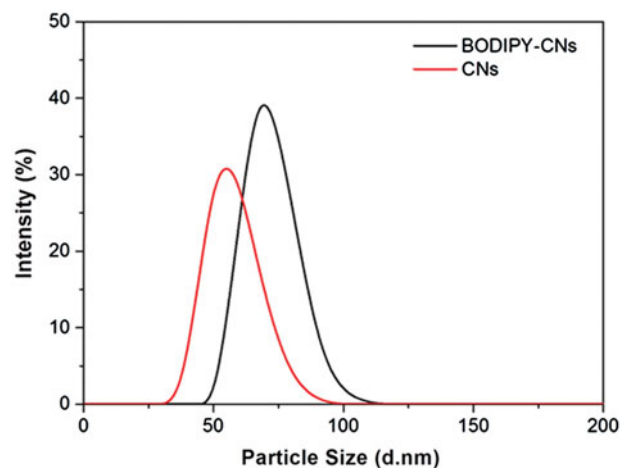


Figure 1. The size distribution by intensity of CN_s and BODIPY-CN_s.

Cell viability assays

MTT assay was performed to evaluate the cell viability of A549 and BEAS 2B cell lines. Cells were seeded on 96-well micro assay plates at a density of 1×10^4 cells/cm² and incubated at 37 °C in 95% air, 5% CO₂ environments for 24 h. BODIPY-CN_s were added and incubated for 24 h, 48 h and 72 h. After incubation, the medium was removed and replaced with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) – containing medium. Plates were incubated for an additional 4 h at 37 °C. MTT medium was removed and 100 μL of DMSO was added to dissolve the formazan crystal. The absorbance was determined using plate reader at a wavelength of 540 nm. Metabolic activity at standard growth conditions was considered 100%. The concentration inhibiting cell viability by 50% (IC₅₀ values) was calculated.

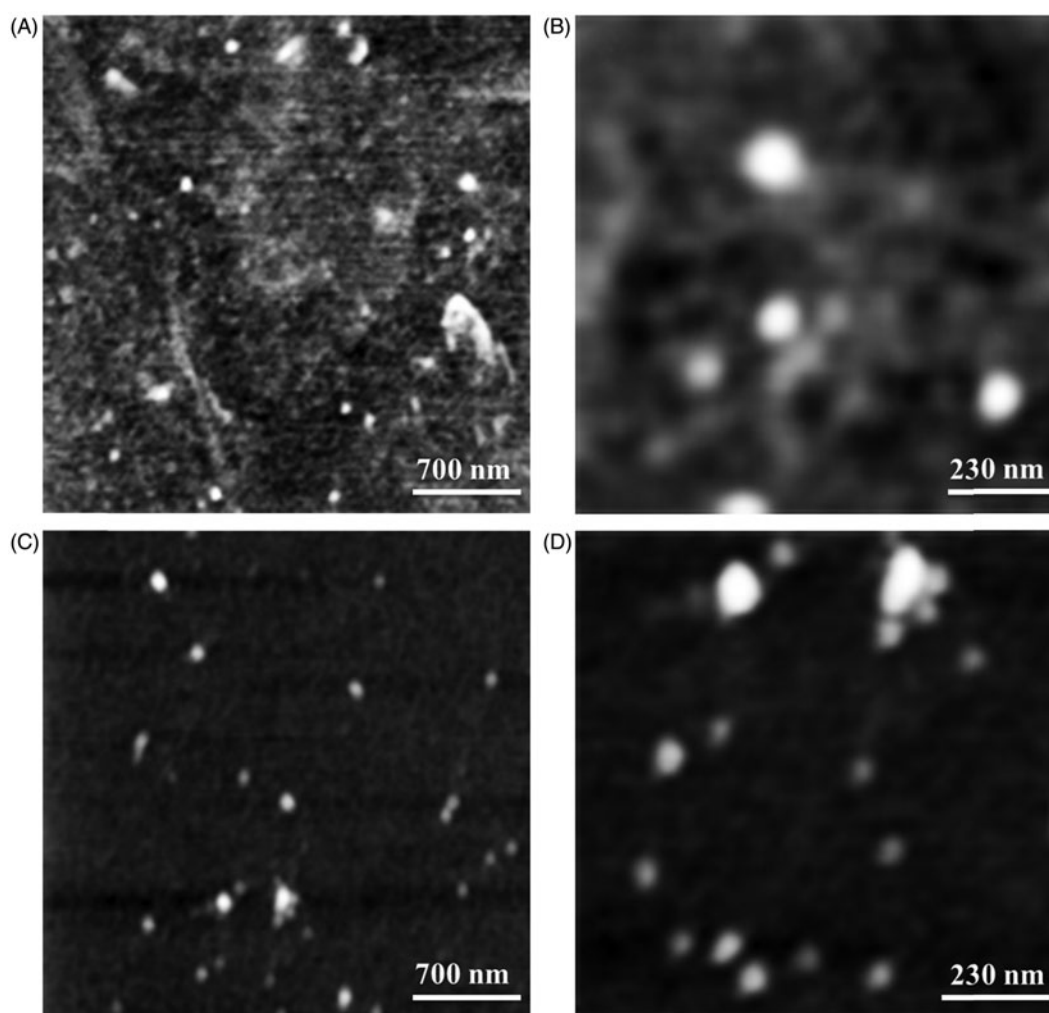


Figure 2. AFM images of (A) CN_s at 700 nm, (B) CN_s at 230 nm, (C) BODIPY-CN_s at 700 nm, (D) BODIPY-CN_s at 230 nm scale.

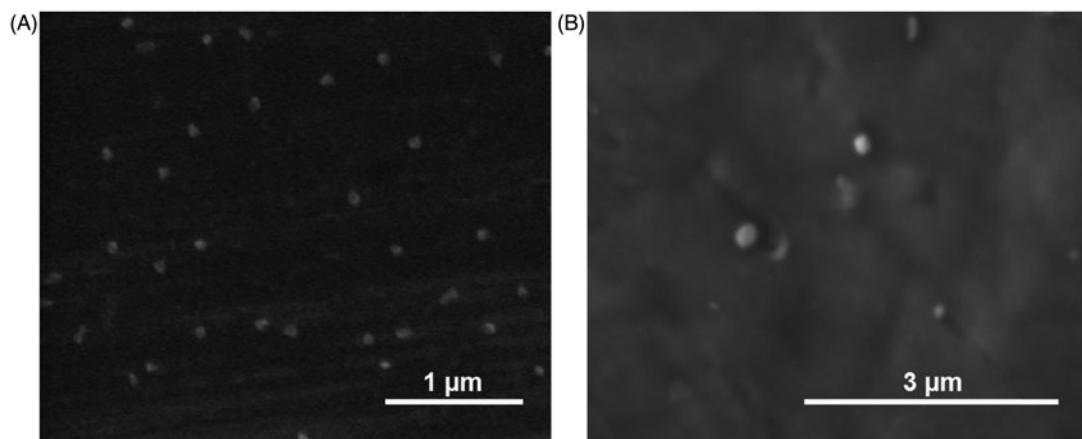


Figure 3. SEM images of (A) CNs at 1 μm and (B) BODIPY-CN at 3 μm scale.

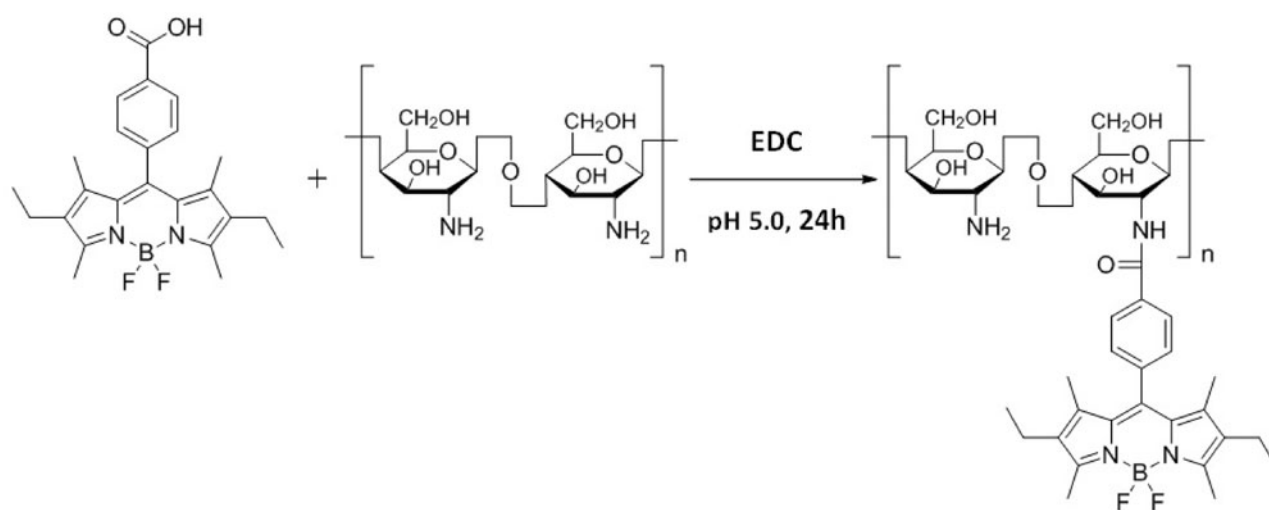


Figure 4. Schematic illustration of the chemical synthesis of BODIPY-COOH conjugated chitosan nanoparticles.

Statistical analysis

The results represented the mean \pm standard deviation from at least three independent experiments. Statistical significance was assessed by One-way ANOVA using GraphPad Prism5, and with the Dunnett's multiple comparison test.

Results and discussion

Preparation and characterization of Ns

Ionic gelation is the convenient method for the fabrication of the chitosan nanoparticles because the process is simple, requires mild conditions and avoids the use of organic solvents and high temperatures (Berger et al., 2004; Nasti et al., 2009). Therefore, in this study, chitosan nanoparticles were synthesized by complexation of polyanionic TPP and cationic chitosan using ionic gelation method at room temperature. The respective average diameters, measured by Zetasizer, of chitosan nanoparticles was about 53.15 ± 6.47 as shown in Figure 1, which was consistent with other reports (Bodnár et al., 2005; Calvo et al., 1997). The polydispersity index (PDI) value of chitosan nanoparticles was 0.287

indicating a narrow size distribution with favorable particle size. When chitosan and TPP were mixed with each other, they spontaneously formed compact nano complexes with an overall positive surface charge. The density of the surface charge is reflected by measured zeta (ζ) potential values, and ζ -potential of prepared CNs were found to be 41.5 ± 1.8 mV and positive due the presence of the protonated amine groups on the chitosan backbone. The morphology and surface appearance of chitosan nanoparticles were nearly spherical shape, uniform and well dispersed as observed by AFM and SEM (Figure 2(A,B) and Figure 3(A)). The size of chitosan nanoparticles was also obtained via AFM in the range of 58–63 nm. The results were consistent with the measurement of Zetasizer. The ability of the ionic gelation process to form chitosan nanoparticles was assessed by employing FT-IR in order to characterize and compare the functional groups of chitosan and chitosan nanoparticles (data not shown). The changes in functional group of chitosan nanoparticle were consistent with our previous studies (İlgü et al., 2011). Therefore, we have concluded that the triphosphoric groups of TPP are linked with ammonium groups of chitosan to form nanoparticles.

Carboxylated BODIPY was synthesized and purified successfully using the method previously described with some modifications (Brizet et al., 2013). The BODIPY-COOH compound was characterized by classical spectroscopic analysis such as NMR spectroscopy and the final product was obtained in 38% yield.

For synthesis of BODIPY-CN_s, the reaction between the chitosan nanoparticles and the BODIPY-COOH was allowed to continue for 24 h on the magnetic stirrer at room temperature. The presence of a large number of primary amine and hydroxyl groups on the chitosan molecule gave a possibility for many chemical interactions. In acidic pH, amino groups of chitosan can undergo protonation and these reactive groups of CN_s on its polysaccharide chains could make easier coupling reactions with BODIPY-COOH fluorophore. The schematic mechanism of BODIPY-COOH and CN_s was illustrated in Figure 4. According to the mechanism, BODIPY-COOH tends to make an interaction with the free ammonium group ($-NH_2$) of CN_s due to the randomly coiled

arrangement form of particles with TPP at low pH during ionic gelation process (Sijumon & Jose, 2010). The BODIPY-COOH amount of the conjugated molecule was measured by UV-visible Spectrophotometry at 510 nm and the binding efficiency (w/w %) of BODIPY-COOH to CN_s was calculated as 95%.

In the present study, the complexation of BODIPY-COOH fluorophore and chitosan nanoparticles to form BODIPY-CN_s were characterized by physicochemical aspects by several methods. The size distribution of BODIPY-CN_s was similar to CN_s itself having a narrow batch with the mean size of about 70.25 ± 11.99 nm. The particle size was slightly larger than chitosan-TPP ones as expected, possibly due to the addition of many BODIPY-COOH fluorophore to CN_s.

The zeta potential of BODIPY-CN_s nanoparticles reflecting the particle surface charge density decreased to $(+) 30.07 \pm 2.6$ mV because of reduced protonated amine groups on the chitosan backbone. This simple relationship could be easily explained as coupling reactions of reactive amino groups of CN_s with BODIPY-COOH fluorophore. The interaction of CN_s with BODIPY-COOH fluorophore could also prevent possible aggregation of chitosan nanoparticles and increase their stability. It was previously demonstrated that the strong inter and intramolecular hydrogen bonding of CN_s could result in aggregation easily (Fan et al., 2012). The carboxyl groups of the fluorophore may form many possible interactions with $-NH_2$ of CN_s to eliminate nanoparticles from aggregation. The morphology of BODIPY-CN_s was visualized by AFM and SEM as shown in Figures 2(C,D) and 3(B). The morphology and surface appearance of BODIPY-CN_s were similar to chitosan nanoparticles. Both AFM and SEM images for BODIPY-CN_s revealed a dense, well dispersed and defined nearly spherical structure. The size of BODIPY-CN_s was measured by AFM images in the range of 70–78 nm, which was consistent with the particle size as measured by DLS. The FT-IR studies of BODIPY-CN_s were also performed to characterize the changes in the chemical structure of chitosan nanoparticles upon binding to the

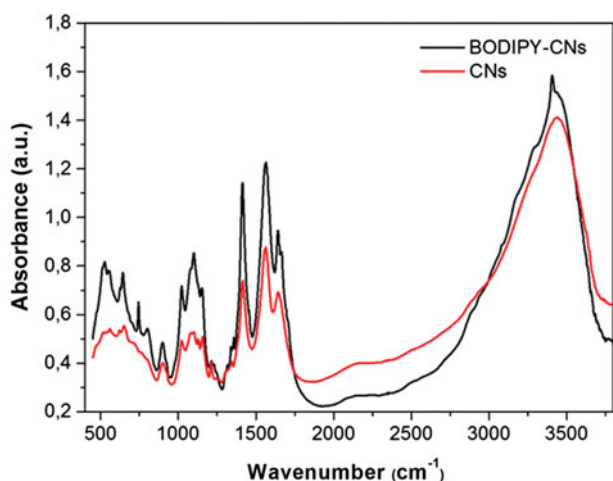


Figure 5. FT-IR spectra of chitosan nanoparticles (CN_s) and BODIPY-COOH conjugated chitosan nanoparticles (BODIPY-CN_s).

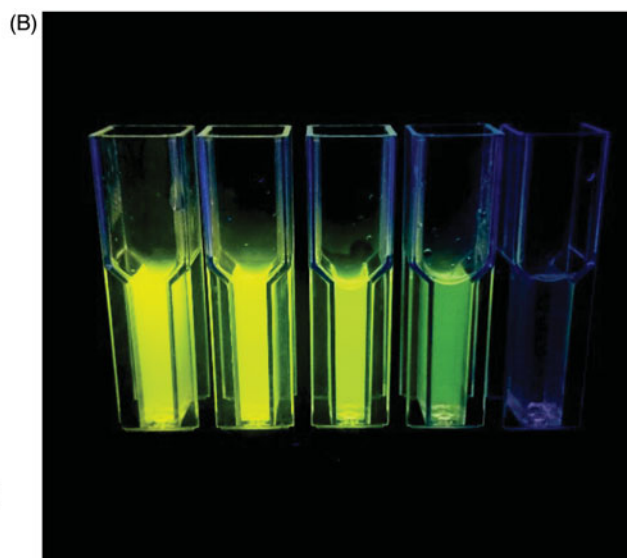
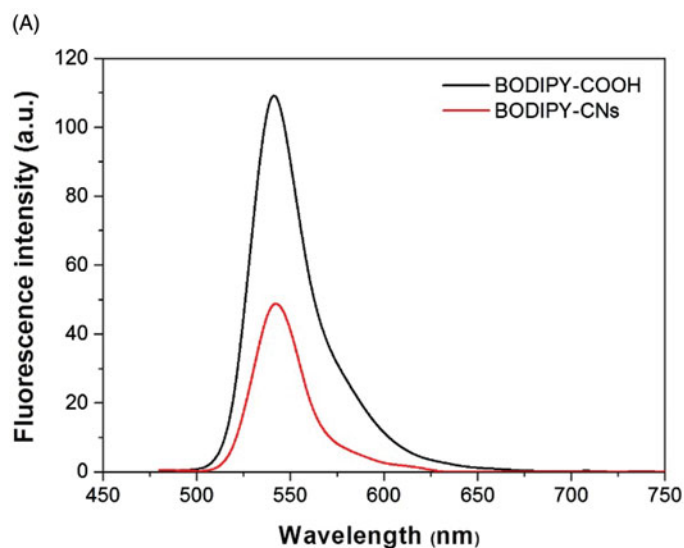


Figure 6. (A) Fluorescence intensities of BODIPY-COOH conjugated chitosan nanoparticles and BODIPY-COOH. (B) Different concentration of BODIPY-CN_s (0.0, 0.05, 0.1, 0.2, 0.5 mg/ml right to left, respectively) under UV light.

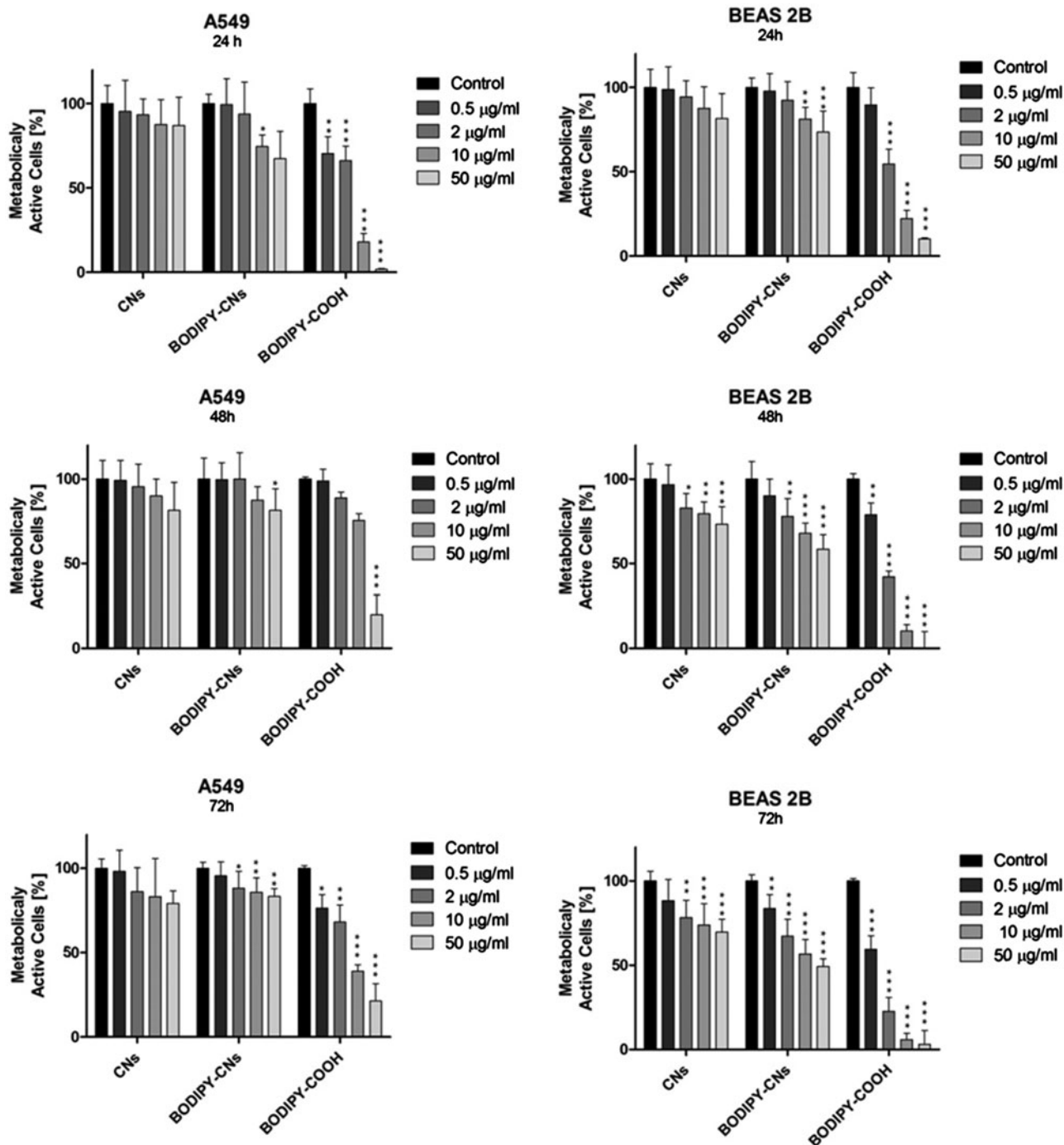


Figure 7. Cell viability of A549 and BEAS 2B cells after the treatment with CNs, BODIPY-CN and BODIPY-COOH for 24 h, 48 h and 72 h. Each value represents the mean \pm SD ($n=6$).

fluorophore (Figure 5). In the spectrum of CNs, a band at 3436 cm^{-1} indicates the combined peaks of the $-\text{NH}_2$ and $-\text{OH}$ group stretching vibration. In the spectrum of BODIPY-CN, a shift from 3436 cm^{-1} to 3440 cm^{-1} was observed, and the band is getting sharper which indicates the hydrogen bonding enhancement. It has been also demonstrated that the enhanced hydrogen bonding could lead to more compact structure due to bonding of BODIPY-COOH to CNs (de Pinho Neves et al., 2014; Qi et al., 2004). The band of CNs at 1642 cm^{-1} corresponds to the CONH_2 and $-\text{NH}_2$ groups. This peak shifted from 1642 cm^{-1} to 1639 cm^{-1} in the spectrum of BODIPY-CN, which shows that the ammonium groups make

an interaction with BODIPY-COOH. This finding also confirms the proposed mechanism for the formation of BODIPY-CN. The peak at 1344 cm^{-1} shifted to 1339 cm^{-1} of BODIPY-CN indicates the existence of CH_3 groups. Both CNs and BODIPY-CN spectrum peaks at 1100 cm^{-1} are attributed to C–O stretching bands.

In the present study, fluorescence properties of BODIPY-CN were determined by fluorescence spectrophotometry (Figure 6A). The results demonstrated that the fluorescent intensity of the chitosan nanoparticles increased due to BODIPY-COOH conjugation. The BODIPY-CN solution in different concentration exhibited a characteristic green

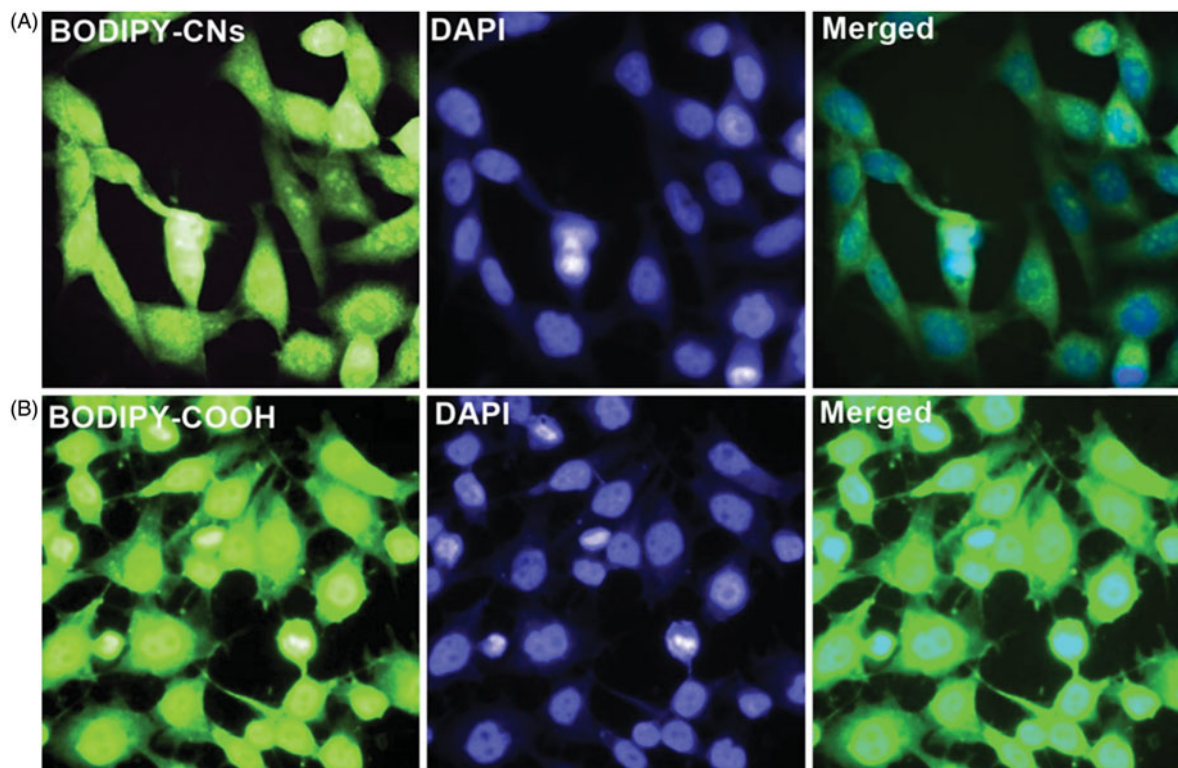


Figure 8. Confocal images of A549 cells treated with 0.2 mg/mL of (A) BODIPY-CNs, (B) BODIPY-COOH after 4 h of incubation at 37 °C.

fluorescence of BODIPY-COOH under UV light (Figure 6B). Although 0.1 mg/ml CNs was in the first tube, it did not show any fluorescence property, the same amount of BODIPY-CNs reflected a considerable fluorescence intensity. As shown in the third tube, 0.2 mg/ml BODIPY-CNs displayed a characteristic green fluorescence color.

***In vitro* cell viability and imaging**

The cytotoxicity of 0.5 $\mu\text{g/mL}$, 1 $\mu\text{g/mL}$, 2 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, 25 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$ concentrations of the BODIPY-CNs, CNs and BODIPY-COOH were investigated on A549 cancer cells and BEAS 2B normal cells as control using the MTT assay (Figure 7).

CNs itself did not show any significant toxic effect on both cells types as expected because chitosan is a natural biopolymer and known as biocompatible. Similar results were obtained in other described reports for chitosan and chitosan-related products (de Campos et al., 2004; Huang et al., 2004). On the other hand, when BODIPY-COOH was applied on A549 and BEAS 2B cells, cell viability decreased with the raise in compound concentration as a concentration dependent manner. After 24 h, BODIPY-COOH-treated cells have $\sim 2\%$ cell viability at 50 $\mu\text{g/ml}$ compound concentration for A549 and 10% for BEAS 2B cells. The present study demonstrated that application of BODIPY-CNs on both cell lines enhanced the cell viability up to 67% for A549 and 74% for BEAS 2B cells. It was also observed that when the incubation time increases up to 72 h, the cytotoxicity of both BODIPY-COOH and BODIPY-CNs was decreased. This finding shows that BODIPY-CNs acts similarly as CNs by lowering cytotoxicity of BODIPY-COOH fluorophore.

Therefore, we can suggest that possible utilization of CNPs together with BODIPY-COOH fluorophore could be a good potential bioimaging agent in various applications in medicine. This new proposed agent would not only act as a fluorescent probe but also be nontoxic for the applied cells.

In order to observe visually the location of BODIPY-CNs in the cells, A549 cancer cells were treated with BODIPY-CNs and blue fluorescent dye 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and then measured by confocal laser scanning microscopy. The present results in Figure 8 show that BODIPY-CNs displayed strong localized fluorescence within the cytoplasm of A549 cells as green color after 4 h of incubation at 37 °C. For the comparison of visual uptake of BODIPY-COOH fluorophore and BODIPY-CNs in A549 cells, similar intense fluorescence signal were observed along a layer on the cell cytoplasm and there was no apparent difference in the fluorescence signal pattern of two compounds treated cells. The cellular internalization and uptake of both dye changed according to varied concentration of BODIPY-CNs and BODIPY-COOH in a dose-dependent manner (data is not shown). Similar results were reported by other studies showing the uptake of the BODIPY-magnetic nanocomposites increases with dose-dependent manner in A549 and Ishikawa cells (Topel et al., 2015). By treatment of cells with BODIPY-CNs, almost whole cells appeared in good shape. Furthermore, similarly, A549 cells were stained with DAPI for the uptake efficiency and localization profile. DAPI as a marker to the nucleus showed blue fluorescence intensity in the nuclei of A549 cells. The merged image reveals that BODIPY-CNs could specifically bind to A549 cells and localize in their cytoplasm.

Conclusions

The present work aims to develop a new fluorescent nanoparticle-based probe for various bioimaging applications that can be used for medical interests. In the development of a multifunctional fluorescent nano-size probe, first, CNs were prepared based on ionic gelation method and BODIPY-COOH fluorescent dye was synthesized as a type of organic fluorescent molecule. Several methods were utilized to determine the physicochemical properties of the CNs and BODIPY-CN. Cytotoxic effects of CNs, BODIPY-COOH and BODIPY-CN were evaluated comparing cell viability of A549 and BEAS 2B cell lines. Confocal images confirmed that the BODIPY-COOH-conjugated chitosan nanoparticles could be utilized efficiently as a multifunctional fluorescent probe. A major advantage of these conjugates is that they are important promising tools for not only in advanced bioimaging systems, but also in intracellular drug delivery, controlled drug release and ultra-sensitive molecular diagnosis events.

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Declaration of interest

The authors declare no conflict of interest.

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