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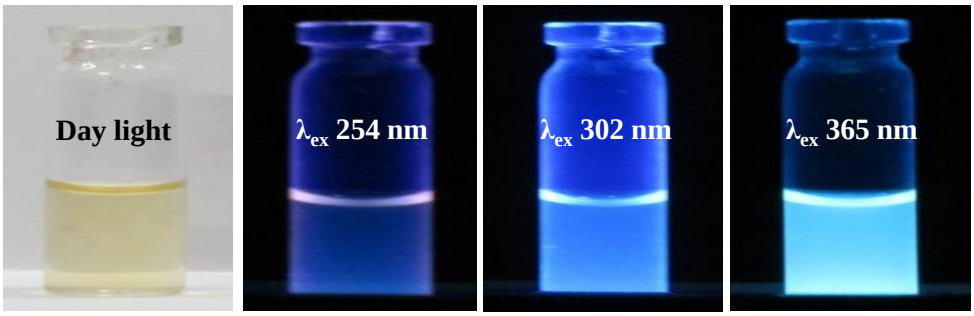
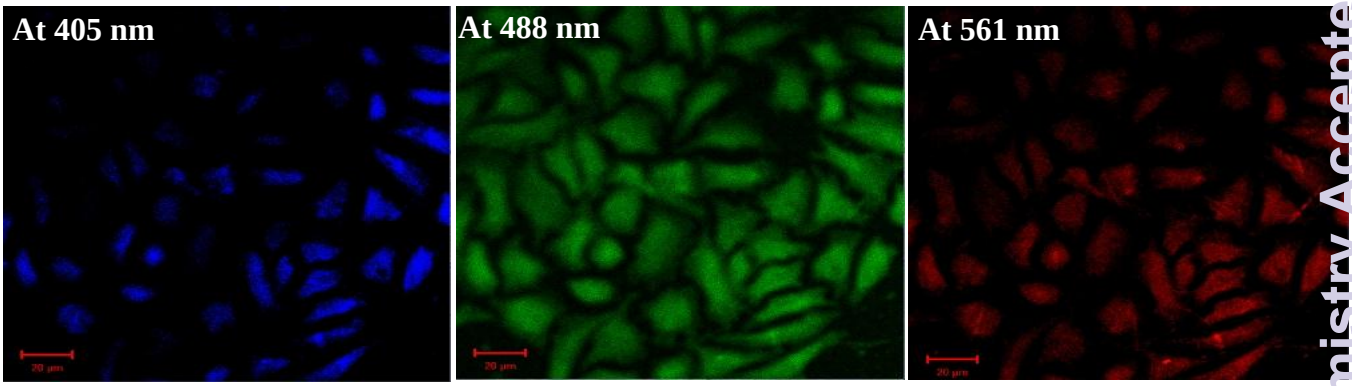


CDs under UV-light



Confocal laser microscopic images of HeLa cells using CDs as fluorescent probes

Preparation of multicolor emitting carbon dots for HeLa cell imaging

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Abstract

We have synthesized biocompatible fluorescent carbon dots (CDs) by one-step hydrothermal method using *Solanum tuberosum* (potato) as a raw material. The CDs were characterized by UV-visible, fluorescence, Fourier transform infrared (FT-IR), X-ray diffraction (XRD), thermogravimetric analysis (TGA), dynamic light scattering (DLS) and high-resolution transmission electron microscopic (HR-TEM) techniques. We found that the carbonization of potato at ~170 °C for 12 h produces highly fluorescent CDs of 0.2 – 2.2 nm size. The synthesized CDs are well dispersed in water and exhibited strong blue and bright blue emissions under UV illuminations (λ_{ex}= 302, 365 nm). The CDs showed strong emission peak at 455 nm when excitation wavelength at 374 nm. The CDs acted as fluorescent probes for multicolor (blue, green and red) imaging of HeLa cells and the CDs did not induce the cell death, which indicates that the CDs are biocompatible and nontoxic to HeLa cells. Therefore, the CDs can be used as probes for cell-imaging applications *in vitro* and *in vivo*.

Keywords: *Solanum tuberosum*, Fluorescence, FT-IR, HR-TEM, DLS and HeLa cells.

1. Introduction

Fluorescence microscopy plays a key role in the current life sciences research. It is regarded as a promising tool for imaging of cells and molecular levels in various biological species in living cells.[1] In this connection, several organic dyes (rhodamines, BODIPY, indocyanines, porphyrines and phthalocyanines) have been used as fluorophores for both *in vitro* and *in vivo* imaging of cells in chemical and biological species.[2] Although these organic molecules are well-defined chemical structures with desired optical and chemical properties, unfortunately these are not ideal agents for multiplexing, long-term, or real-time imaging of cells. Since they have exhibited some drawbacks such as poor solubility, and inherent photophysical properties (low photobleaching thresholds, broad absorption/emission spectra, and small Stokes shifts).[3,4] To overcome the above problems, the integration of nanoscience with fluorescence microscopy has attracted growing attention in biomedical research for imaging live cells, tissues and entire organisms.[5,6] Ideally, fluorescence nanoparticles exhibit unique physico-chemical (excellent dispersibility and stability, high targeting selectivity, high photostability, good biocompatibility and large stock shift) and optical (high quantum yield, size-dependent absorption and emission) properties, which makes them as ideal fluorescent probes in the development of new nanoprobe-based tools for bioimaging, diagnostics, drug delivery and therapy.[7-9]

In recent years, carbon dots have proved to be a new class of discrete that contains small carbon nanoparticles with less than 10 nm size.[10] Compared to heavy-metal-based quantum dots, carbon nanoparticles exhibit excellent photostability, biocompatibility, good water dispersability, and low toxicity, and have shown great advantage in cell

imaging.[11] In addition, CDs exhibited bright up conversion photoluminescence, size dependent photoluminescence and multicolor emission due to their surface-passivation by organic or biomolecules.[12] Importantly, the CDs show fluorescence in the red or NIR range, which makes them as potential fluorescent probes for bioimaging.[13] Due to their fascinating properties, many simple and effective synthetic approaches such as laser ablation, arc discharge, combustion, thermal treatment, electrochemical oxidation, hydrothermal, ultrasonic treatment and microwave methods have been described for the preparation of fluorescent CDs by using various materials as carbon source.[14-21] To meet "green chemistry" approach, many efforts have been devoted on the development of green chemistry-based methods for preparation of fluorescent CDs using eco-friendly chemicals or natural precursors as raw materials.[15] As a result, a variety of eco-friendly fluorescent CDs with the size smaller than 10 nm have been prepared by using banana,[22] orange,[23] food waste,[24] pomelo peel,[25] coffee grounds,[26] watermelon peel,[27] *Trapa bispinosa* peel,[28] bread, sugar, jaggery,[29] gelatin,[30] low-cost organic chemicals,[31-33] and pipe tobacco[34] as raw materials. These fluorescent CDs have successfully used as nanoprobes for sensing of molecular species and imaging of various cells. Moreover, the CDs were prepared by ionic liquid-assisted electrochemical exfoliation of graphite electrode, and developed as photoluminescence and electrochemiluminescence probes for efficient detecting $Ru(bpy)_3^{2+}$ in the solution.[35] Similarly, CDs were functionalized with branched poly(ethylenimine) and used as a fluorescent probe for detection of Cu^{2+} ions in water samples.[36]

Herein, we have developed a simple method to prepare multi-color emission CDs and used as a fluorescent probes for HeLa cell imaging. Figure 1 shows the synthetic

process for preparation of CDs using *Solanum tuberosum* (potato) as a raw material. The CDs were prepared without use of severe synthetic conditions such as strong solvents, surface passivation reagents, and a complicated post-treatment process, which follows green chemistry principles. Importantly, we further demonstrate that these CDs act as very effective fluorescent probes for HeLa cells imaging.

2. Experimental

2.1. Materials and chemicals

Solanum tuberosum (potato) was purchased from the local supermarket, Surat, India. Dulbecco's Modified Eagle Medium (DMEM) and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich, USA. Dichloromethane, paraformaldehyde, glycerol and dimethyl sulfoxide were purchased from Merck Ltd., India. HeLa cells were purchased from National Centre for Cell Science, Pune, India. All chemicals were of analytical grade and used without further purification. Milli-Q-purified water was used for sample preparations.

2.2. Preparation of the CDs from Solanum tuberosum

Solanum tuberosum was used as a carbon source for the preparation of water dispersible CDs *via* hydrothermal treatment. Briefly, 400 gm of *Solanum tuberosum* was grinded and the obtained liquid was mixed with 500 mL of water using an electrical hand blender. The above solution was filtered through the vacuum to obtain pulp free product. Then, 500 mL of solution (pulp-free) was transferred into a 700 mL Teflon-lined stainless-steel autoclave and heated at 170 ^{o}C for 12 h until a dark brown solution

formed. The resulted dark brown solution was filtered through 0.45 μm filter paper to remove large particles and then washed with dichloromethane to remove unreacted organic moieties. The filtrate was centrifuged (15,000 rpm, 20 min), and the resultant supernate was dialyzed against deionized water for 12 h. The CDs dispersed solution was stored at 4 ^{o}C and used as stock solution for further characterization and cell imaging.

2.3. The CDs as probes for HeLa cells imaging

To culture human epithelial carcinoma (HeLa) cells, DMEM with 10% fetal bovine serum and 1% penicillin streptomycin with 5% CO_2 were used as a media and the cell culture was carried out at 37 ^{o}C. Before cell imaging, the cells were pre-cultured with the above media (0.5 mL), mixed with 20 μL of CDs (10 μg/mL) and incubated for 2 h. The cells were washed thrice with PBS buffer, fixed by 4% paraformaldehyde and then mounted using 50% glycerol. The CDs-conjugated HeLa cells images were measured by confocal laser scanning microscopy (Carl Zeiss 510 LSM, Jena, Germany) at laser excitations 405, 488 and 561 nm.

2.4. Cytotoxicity of CDs

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) based assay was used for the investigation of CDs cytotoxicity towards HeLa cells. Initially, HeLa cells were seeded in a 96-well U-bottom plates (90 μL·well^{-1}) for 12 h. The HeLa cells were incubated with different concentration of CDs (25, 50, 100, 200, and 500 μg/mL) for 24 h. The cells were incubated with freshly prepared MTT solution (50 μL, 5 mg/mL) for 4 h. The culture medium was discarded, the CDs-conjugated cells were treated with 150 μL

of DMSO and then vortexed for 15 min in dark at room temperature. Their optical density (*OD*) was measured at 570 nm using Microplate reader. The cell viability was calculated by using following equation

$$\text{Cell viability (\%)} = OD_{\text{treated}} / OD_{\text{control}} \times 100\%$$

where OD_{control} was obtained in the absence of CDs, and OD_{treated} was obtained in the presence of the CDs.

2.5. Quantum yield measurement

The quantum yield of the CDs was calculated from the equation[37]

$$Q_{CDs} = Q_R \cdot \frac{I_{CDs}}{I_R} \cdot \frac{A_R}{A_{CDs}} \cdot \frac{\eta_{CDs}^2}{\eta_R^2}$$

In here "CDs" and "R" refer to sample and reference respectively. "Q" means Quantum yield. 'I' is the intensity of fluorescence spectra, 'A' is the absorbance at excited wavelength and 'η' is the refractive index of the solvent (using quinine sulfate-quantum yield 54% in 0.1 M of H_2SO_4 as the reference). The "A_{CDs}" denotes the absorbance of CDs, and "A_R" represents the reference that is used in this equation. The quantum yield of CDs was measured at an excitation wavelength 360 nm and quantum yield was found to be 6.14%.

2.6. Instrumentation

UV-visible spectra were measured by using Maya Pro 2000 spectrophotometer (Ocean Optics, USA). The fluorescence spectra were recorded using a RF-5301 PC Shimadzu spectroflourometer. FT-IR spectra were recorded on a Perkin Elmer (FT-IR spectrum BX, Germany). Fluorescence lifetime was measured using Horiba Jobin Yvon

IBH Fluorocube instruments after exciting 370 nm picosecond diode laser. The morphology and microstructure of CDs were examined by JEOL-HR-TEM 3010 with an accelerating voltage of 200 kV. The HR-TEM samples were made by dropping an aqueous solution onto a 300-mesh copper grid coated with a lacy carbon film. The CDs XRD profiles were recorded on a Rigaku diffractometer (Rigaku, Japan) equipped with graphite monochromatized CuKα (λ=0.15405 nm) radiation in the range of 10° to 70°. Thermogravimetric analysis were carried out on a thermal analyzer (model: SDT Q600, TA Instruments) by heating sample (5 mg) from room temperature to 700 ºC (20ºC min^{-1}) under N_2 gas atmosphere. DLS measurements were carried out on a Zetasizer Nano ZS90 (Malvern, UK). Cell images were measured using Carl Zeiss 510 LSM laser scanning confocal microscope. The elemental analysis was performed by using Flash 2000 CHN analyzer (Thermo Scientific, USA).

3. Results and discussion

3.1. Synthesis and characterization of CDs

Solanum tuberosum contains carbohydrates, vitamins, minerals, as well as an assortment of phytochemicals (carotenoids and natural phenols). In this work, the CDs were synthesized from *Solanum tuberosum* juice by hydrothermal treatment at 170 ºC for 12 h. The crude product was centrifuged at 15000 rpm and further dialyzed against distilled water to obtain water dispersible CDs. The prepared CDs are highly dispersible in water because of enriched organic functional groups (carbonyl, carboxylic and hydroxy) from the carbonization of potato, which facilitates to use them as fluorescent probes for biomolecules in aqueous media. It can be observed that the CDs show strong

bluish fluorescence under UV illumination at 365 nm, which allows to use them as probes for cell imaging (Figure 1). Figure 2a shows the UV-vis and photoluminescence (PL) spectra of the CDs. It can be noticed that the UV−vis absorption spectrum shows two UV absorption bands at 245 and 310 nm, which could be attributed to the π→π* transition of C=C and to the n→π* transition of C=O in the CDs.[38] Upon excitation of the CDs at absorption band of 374 nm, the PL spectrum shows a strong peak at 455 nm with a full width at half maximum (FWHM) of 97.04 nm, which is similar to that of the carbon particles derived from β-cyclodextrin.[33] In order to investigate the optical properties of the CDs, we recorded the optical images of the CDs under UV illuminations at 254, 302 and 365 nm (Figure 2b). It can be observed that the CDs exhibited blue and bright blue emissions under UV illuminations at 302 and 365 nm, which can be observed with naked eye. Interestingly, we observed that the property of excitation-independent emissions is quite different from our recent report[18] and the previous reports on CDs.[27-28,38] This is due to the presence of organic functional groups (carbonyl, carboxylic and hydroxy) in CDs that are derived from carbohydrates, vitamins, minerals, and phytochemicals (carotenoids and natural phenols) *via* carbonization of potato. We also observed that the prepared CDs are very stable for several months without any floating or precipitated nanoparticles.

From the CDs dispersion, a strong PL emission peak centered at 455 nm (FWHM, 97.04 nm) is recorded under excitation at 374 nm (Figure 3). To further investigate optical properties, PL emission spectra of the CDs are measured at various excitation wavelengths. As shown in Figure 3b, with the increase of excitation wavelength from 300 to 540 nm, emission peaks are red-shifted from 380 to 540 nm, while the PL intensities are decreased remarkably, confirming that the similar luminescent properties of carbon

nanoparticles that are derived from glucose and sucrose. Since, the CDs exhibit an excitation-dependent emission, which is an intrinsic property of the CDs.[39] These optical properties are mainly due to (i) different size (quantum effect), (ii) different distributions of emissive trap sites on each CDs, (iii) radiative recombination of excitons, and (iv) free zigzag sites with a carbene-like triplet ground state. The fluorescence properties of CDs always depend on the size and presence of organic functional groups in carbon source, which influences the excited state energy distribution, the charge separation, and/or the confinement of electrons and holes in the CDs and their radiative recombination.[13,22-24] Furthermore, we also studied that the time-resolved fluorescence decay of the CDs. As shown in Figure 3c, the fluorescence lifetime of the CDs is very short and reflected a fast exciton recombination process. The fluorescence decay was well fitted with the best three-exponential function, indicating that an ultrafast electron transfer process can be achieved in nanoseconds, which makes them as potential candidates in bioimaging. We observed that the lifetimes of CDs are τ_1= 3.93 ns, τ_2= 0.92 ns, τ_3= 12.12 ns and the mean lifetime is found to be 5.65 ns, which is very closer values in the literature reports.[30,40] Therefore, the prepared CDs showed good ability to emit different colors (blue, green and red) at different excitation wavelengths, which allows them to act as fluorescent probes for cell imaging.

In order to identify the functional groups and to determine the chemical composition of the CDs, we studied that the FT-IR, TGA, XRD and CHN data of the CDs. As shown in Figure 4a, the CDs shows the characteristic peaks at 3279 cm^{-1} and 1747 cm^{-1} corresponding to the stretching vibrations of -OH and C=O bonds, respectively.[41] The peak at 1651 cm^{-1} corresponds to C=C stretching of polycyclic

aromatic hydrocarbons, indicating that the presence of sp^2 hybridization.[42] Moreover, the peaks centered at 1369 cm^{-1} and 1099 cm^{-1} are ascribed to asymmetric and symmetric stretching vibrations of C–O–C in the carboxylate groups.[43,44] The peaks at 2877 cm^{-1} and 820 cm^{-1} are attributed to the C−H stretching mode and C−H out-of-plane bending mode of CDs. The C–N stretching was observed at 2360 cm^{-1}. Furthermore, the peaks at 1226 cm^{-1}, 1180 cm^{-1} and 1060 cm^{-1} corresponded to the stretching and bending vibrations of C-O bond in the CDs, suggesting that the partial oxidation of CDs surfaces.[45] The above data suggests that the synthesized CDs have functionalized with hydroxyl, alkyl, carboxylic and carbonyl groups, which are derived from organic molecules in potato. To confirm the surface functionalization of CDs, TGA analysis was performed in two stepwise weight-losses at 149 and 560 ^{o}C. As shown in Figure 4b, the initial weight loss of 5-6% at 149 ºC is due to evaporation of water molecules and other molecules attached *via* weak hydrogen bonding within the CDs.[46] Further a constant weight loss of 37% until 560 ºC may be attributed to the decomposition of organic functional groups in CDs.[28] Figure 4c shows the XRD patterns of the CDs. The four Bragg reflections at 28.28º, 40.44º, 50.12º and 58.56º were assigned to the diffractions of (002), (101), (102) and (103), which confirm that the presence of turbostratic and graphitic carbons in the CDs.[47] The sharp peak at 28.28° corresponds to the inter-chain distance of densely packed alkyl chains in CDs. Table 1 shows the elemental composition of CDs by CHN analyzer, which confirms that the CDs contain C, O, N, and S elements. The morphology and size of the CDs were investigated by HR-TEM and DLS. As shown in Figure 5a-c, the CDs are nearly monodispersed quasi-spherical crystals with an

average dimension of 1.0 nm. Similarly, the DLS data indicated that the CDs are in monodispersion state with an average hydrodynamic diameter of ~ 2.15 nm (Figure 5d).

3.2. The CDs as multicolor emitting probes for HeLa cells imaging and cell viability

Before testing the applications of CDs as fluorescent probes for HeLa cell imaging, we studied that the photostability of CDs solution under UV illumination (at 365 nm). As shown in Figure 6, photobleaching did not observe and the fluorescence remains intact even after excitation wavelength at 365 nm for 120 min, indicating that the CDs exhibit good photostability under UV light. We have investigated their performance as fluorescent cell labels by incubating HeLa cells with the CDs (10 μg/mL) for 2 h. After incubation, the cells were washed and measured their optical properties by fluorescence microscopy. The microscopic images of CDs-conjugated HeLa cells were recorded at different excitation wavelengths (405, 488, and 561 nm). As shown in Figure 7a, there is no detectable emission signal from HeLa cells (without CDs) at bright field. However, the HeLa cells are became brightly illuminated multicolor (blue, green and red) when imaged on the fluorescence microscope at excitation wavelengths 405 (blue), 488 (green), and 561 nm (red), respectively (Figures 7b-d). Moreover, we also investigated that the confocal fluorescence microscopic images of the CDs-conjugated HeLa cells in fluorescence mode only (without bright field) at excitation wavelengths 405 (blue), 488 (green), and 561 nm (red), respectively (Figures 7e-g). These results demonstrated that the CDs are successfully acted as probes to entrap the endosomes, multivesicular bodies, and lysosomes in HeLa cells through carbon nanoparticles endocytosis, which are responsible for the pockets of the CDs intensity and a localized CDs intensity

distribution.[48] Importantly, the prepared CDs are hydrophilic in nature and having many active functional groups (carbonyl, hydroxy and carboxylic acid), which allows easy internalization of the CDs into the cytosol as well as in nuclear compartments with a broad distribution of fluorescence nanoparticles. It can also be observed that the fluorescence signals from the CDs are visible in the cytosol, and localized in endosomes or lysosomes, indicating that the CDs can well point out the endosomal/lysosomal positions, and the CDs can be efficiently internalized by the cells. This result indicates that the CDs exhibit good ability to enter into cytosol *via* multiple interactions with cytoplasmic proteins, suggesting that the CDs can act as nanocarriers for drug delivery. Moreover, the laser confocal microscopic images also suggested that fluorescence intensity of labeled HeLa cells remains stable for 1 h (after constant excitation), indicating that the good photostability and low photobleaching of CDs. Therefore, the CDs acted as potential candidates for specific HeLa cell labeling and imaging with multicolors.

Considering the utility of the CDs as fluorescent probes for cell imaging, it is fascinating to explore their toxicity towards HeLa cells. Therefore, the cytotoxicity of the CDs is further tested by MTT assay. As shown in Figure 8, the HeLa cell viability is more than 100% when the CDs concentration at 25 μg/mL, however, the cell viability decreased from 100 % to 95% when the CDs concentration is increased from 50 to 200 μg/mL, which indicates that the CDs did not induce the HeLa cell death. It is worth to mention that the CDs did not exhibit any cytotoxicity towards cells and are tolerable even at high concentration (500 μg/mL), which confirms that the CDs are cytocompatible and not induced cell death and survival rates greater than > 95%. These results demonstrated

that the CDs are successfully acted as probes for imaging of cells without any cytotoxicity and can be used for *in vitro* and *in vivo* applications.

4. Conclusions

In summary, we have demonstrated a facile and one-pot hydrothermal method for the synthesis of carbon dots by using low-cost green material (*Solanum tuberosum*) as a carbon source. HRTEM results revealed that the carbon dots are well dispersed in water with a narrow size distribution of 0.2 nm -2.2 nm. The prepared carbon dots exhibited strong blue and bright blue emission under UV light (λ_{ex}= 302, 365 nm) and showed quantum yield up to 6.14%. The carbon dots are used as promising multicolor (blue, green and red) fluorescent probes for HeLa cell imaging. The carbon dots show efficient intracellular distribution ability and found mainly in the cytoplasm region of the cells. The carbon dots did not induce HeLa cell death even at the concentration of 500 µg/mL, which indicates that the carbon dots are biocompatible in nature and used a promising material for both *in vitro* and *in vivo* applications.

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Figure captions

Figure 1. Schematic representation for facile synthesis of the CDs using *Solanum tuberosum* as a raw material.

Figure 2. (a) UV-visible absorption and fluorescence emission spectra of the CDs. Inset picture shows the CDs under daylight (left) and UV light at 365 nm wavelength (right) (b) Photographic images of CDs under UV illuminations at 254, 302 and 365 nm.

Figure 3. (a) Fluorescence spectra (excitation spectra: black line and emission spectra: red line) of the CDs (b) Fluorescence emission spectra of the CDs obtained at different excitation wavelengths progressively increasing from 300 to 540 nm with a 20 nm increment (c) the fluorescence decay (life time) curve of the CDs upon excitation wavelength at 374 nm.

Figure 4. (a) FT-IR spectrum, (b) TGA analysis and (c) XRD pattern of the CDs.

Figure 5. HR-TEM images of the CDs at different magnification (a) 20 nm and (b) 10 nm (c) particle size distribution histogram of the CDs and (d) DLS of the CDs.

Figure 6. Photostability study of the CDs under UV light irradiation. The CDs solutions were irradiated by UV light at excitation wavelength of 365 nm up to 120 min at different time interval.

Figure 7. (a) Confocal laser microscopic images of HeLa cells without CDs. The confocal laser microscopic images of HeLa cells after the cellular uptake of carbon dots in bright field and fluorescence mode at excitation wavelengths (b) 405 (blue), (c) 488 (green), and (d) 561 (red) nm. Confocal laser microscopic images of HeLa cells after the cellular uptake of carbon dots at excitation wavelengths (e) 405 (blue), (f) 488 (green),

and (g) 561 (red) nm, without bright field. Scale bar indicates 20 μm. The concentration of the CDs is 10 μg/mL.

Figure 8. MTT based cytotoxicity assay of HeLa cells after 24 hrs incubation with different concentration of CDs (25, 50, 100, 200 and 500 μg/mL).

Table 1. The elemental, Q and life-time measurement (τ) analysis results of CDs.

Sample	C%	H%	N%	S%	O% (Calculated)	Q%	Lifetime (ns)	Hydrodynamic diameter (nm)
CDs	48.8	4.48	1.94	0.59	44.19	6.14	5.65	2.15

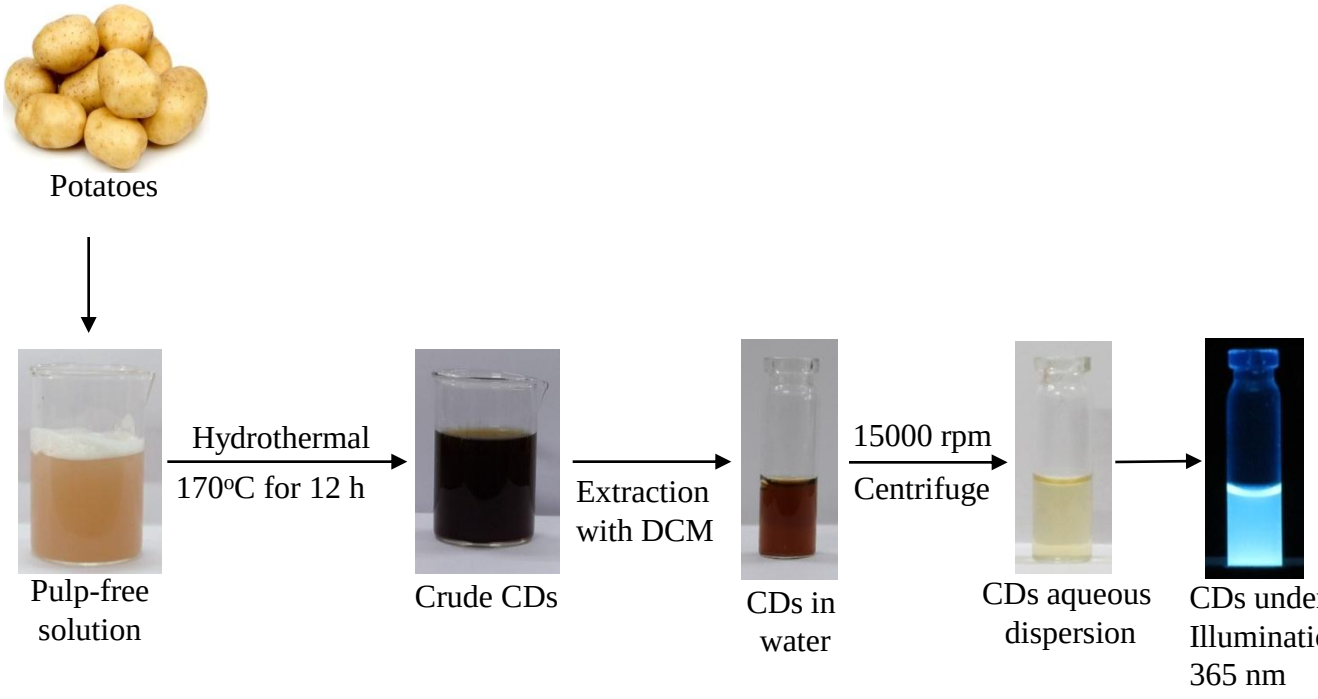
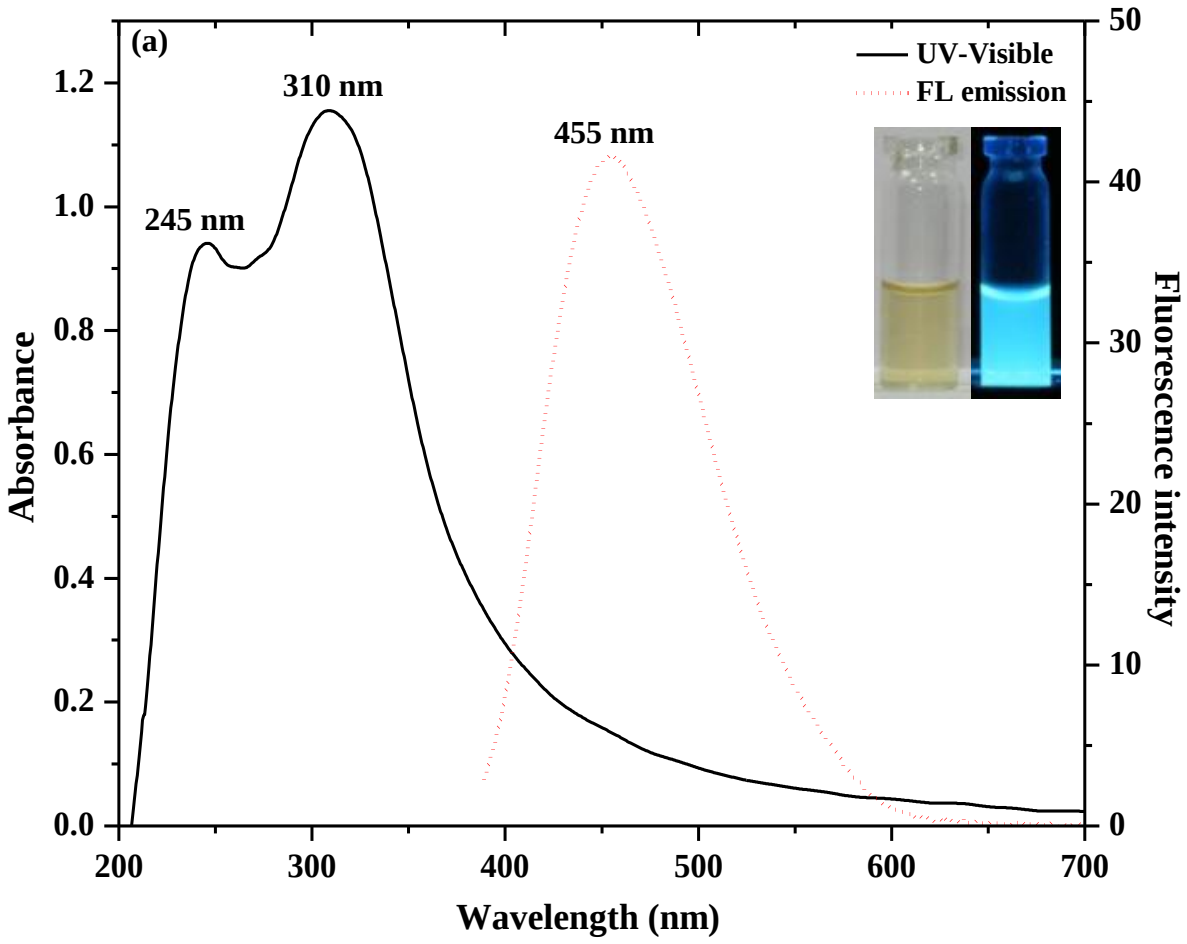
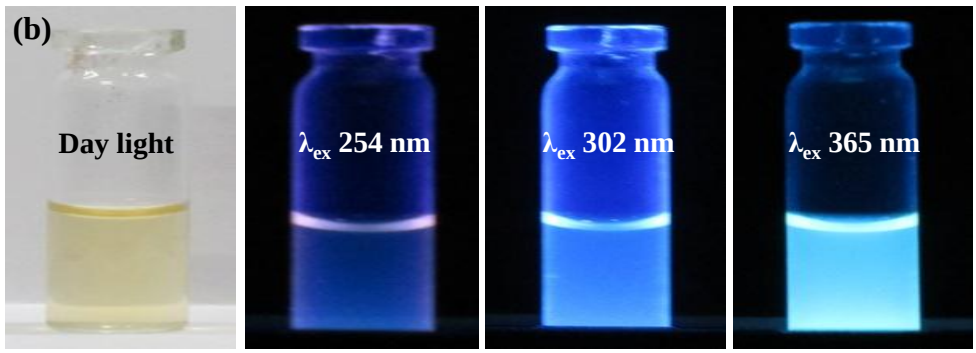


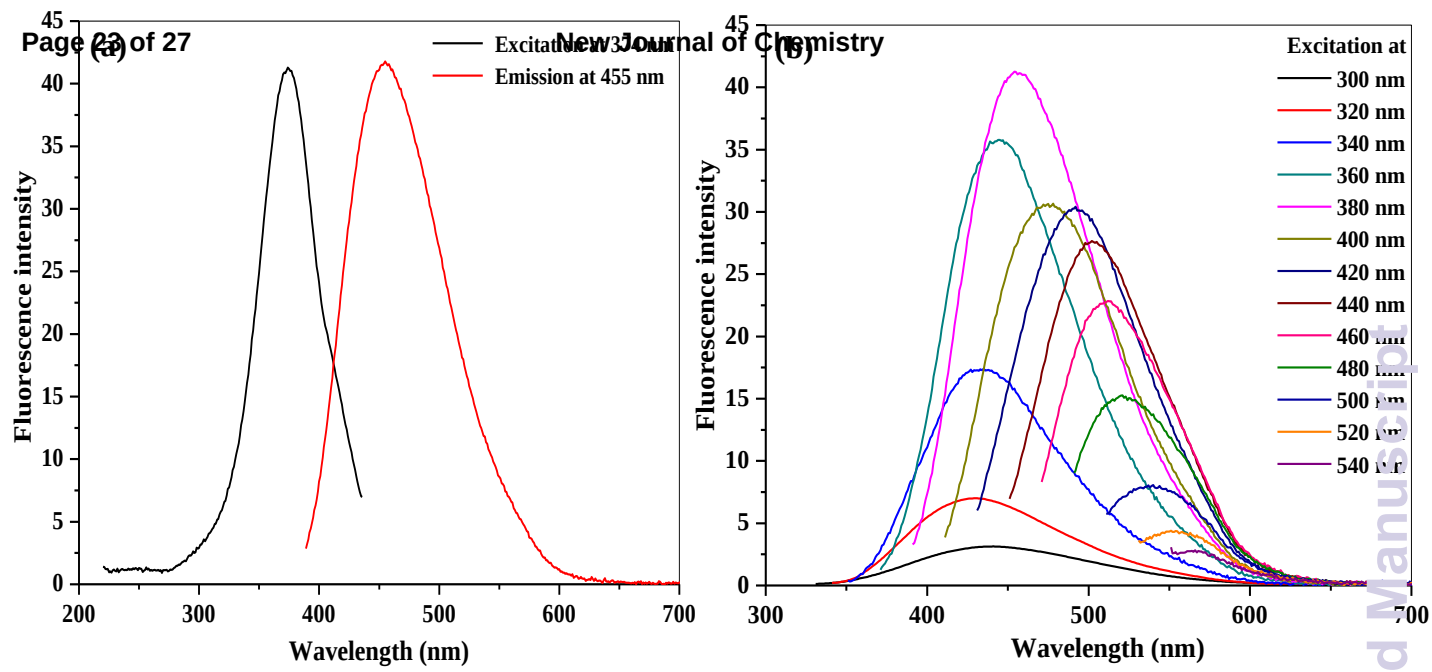
Figure 1





Under UV-light illuminations

Figure 2



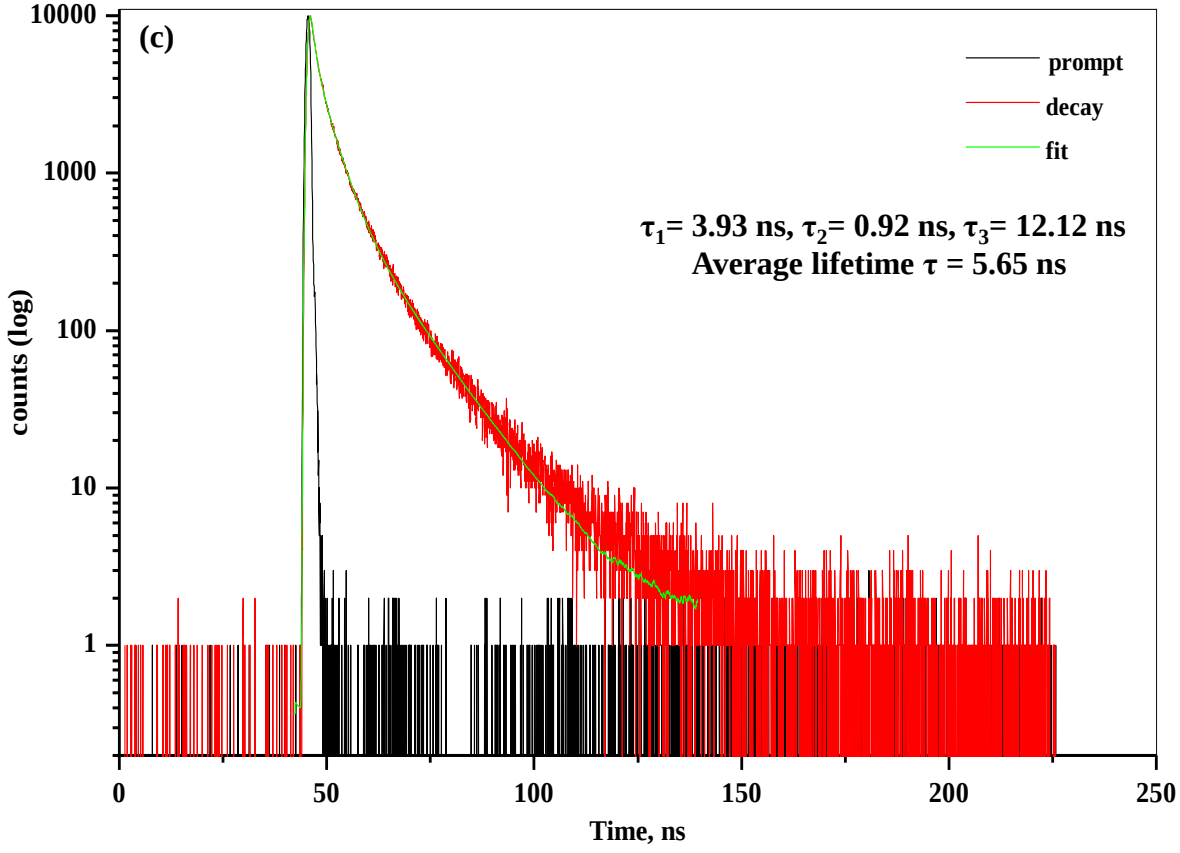
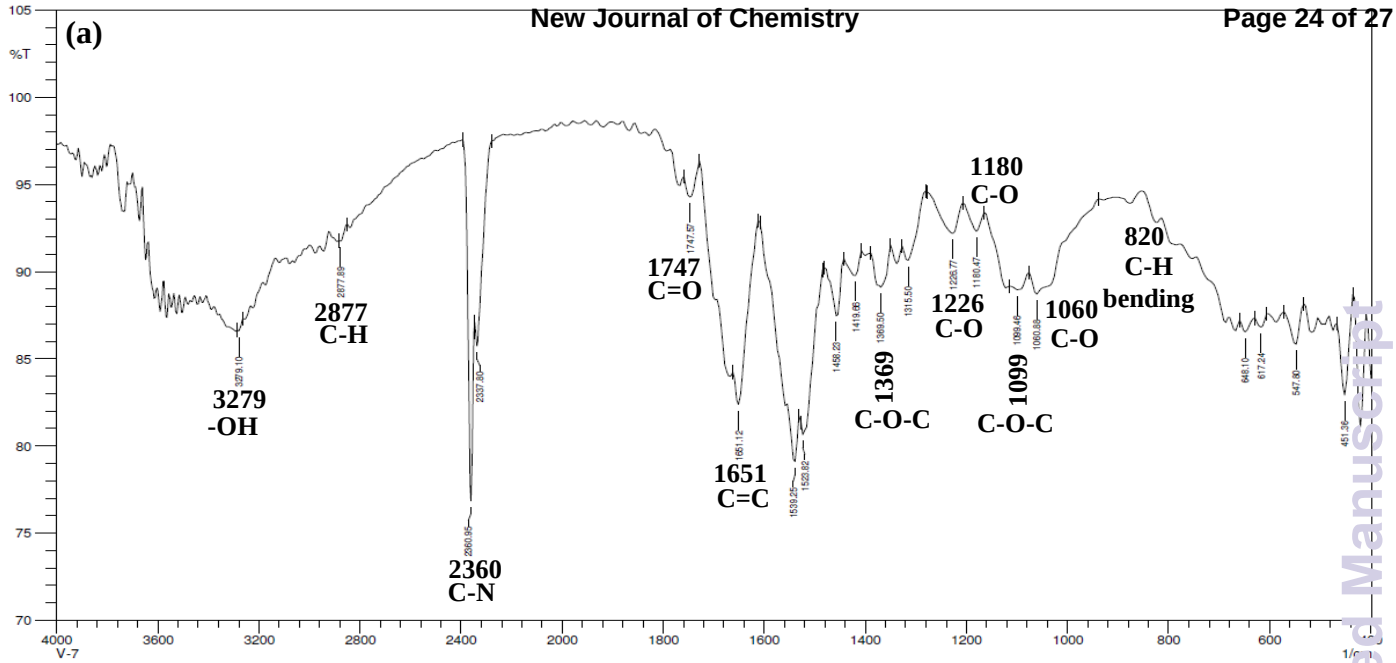
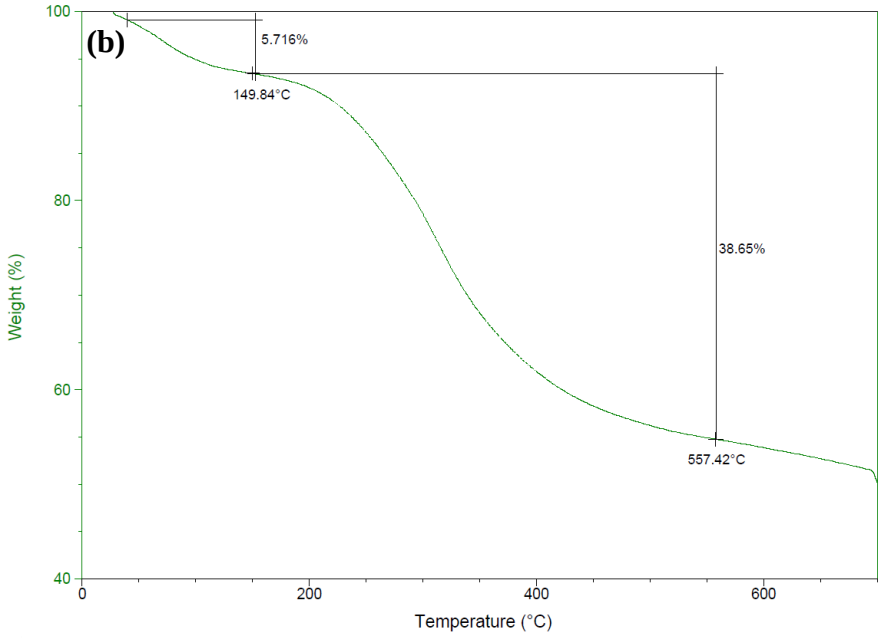


Figure 3





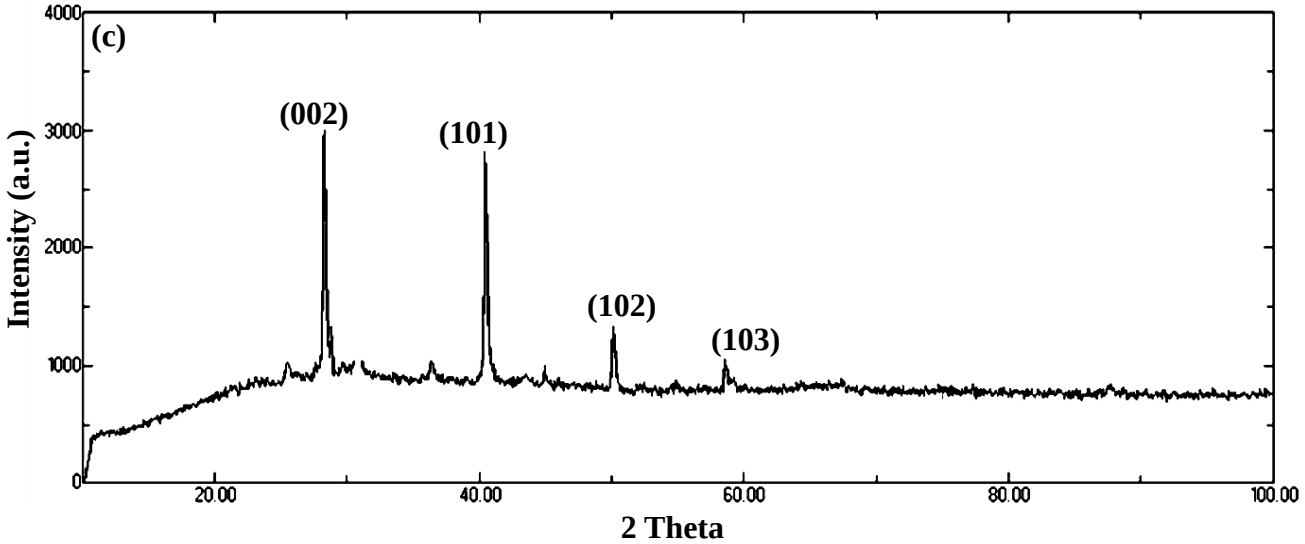


Figure 4

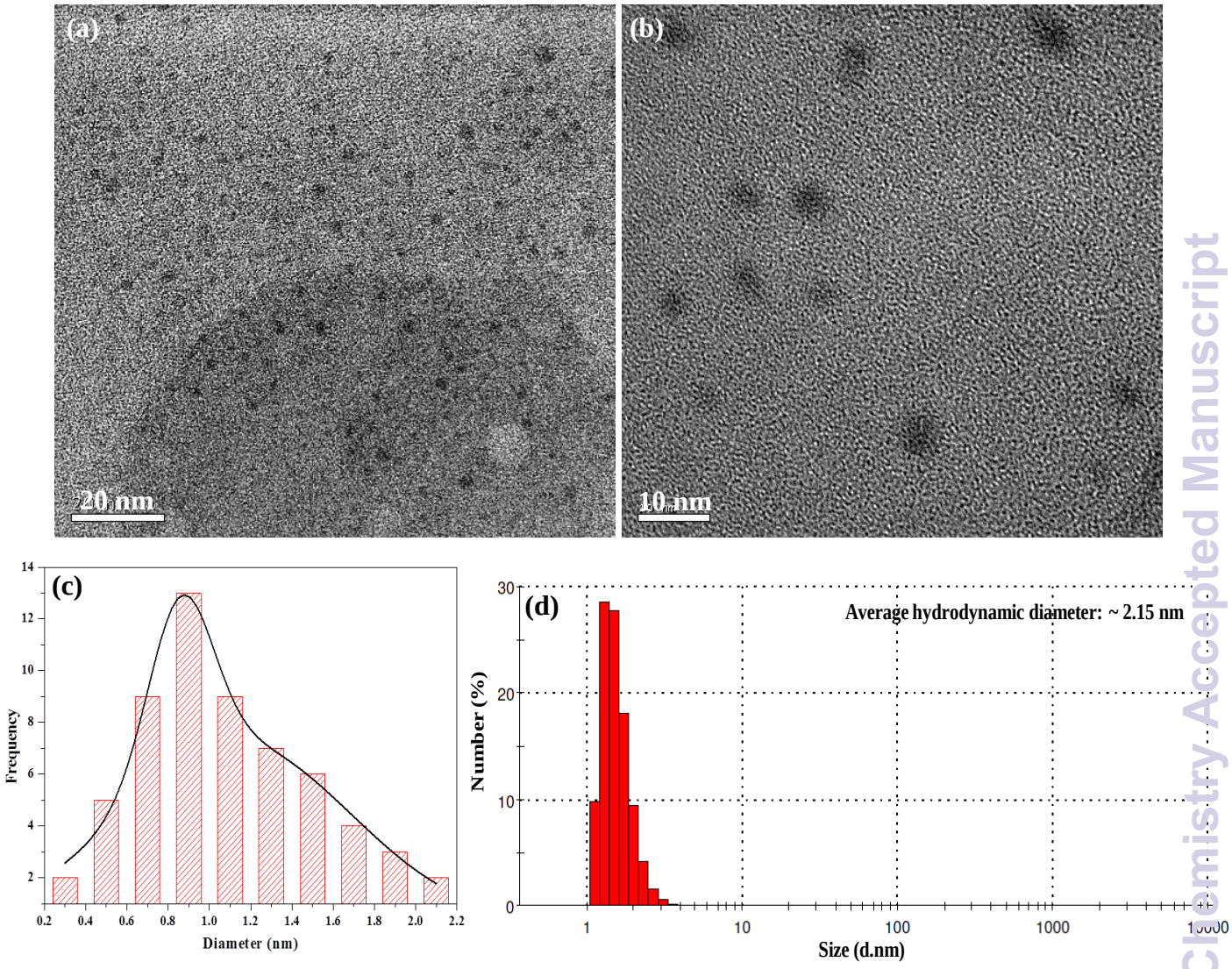
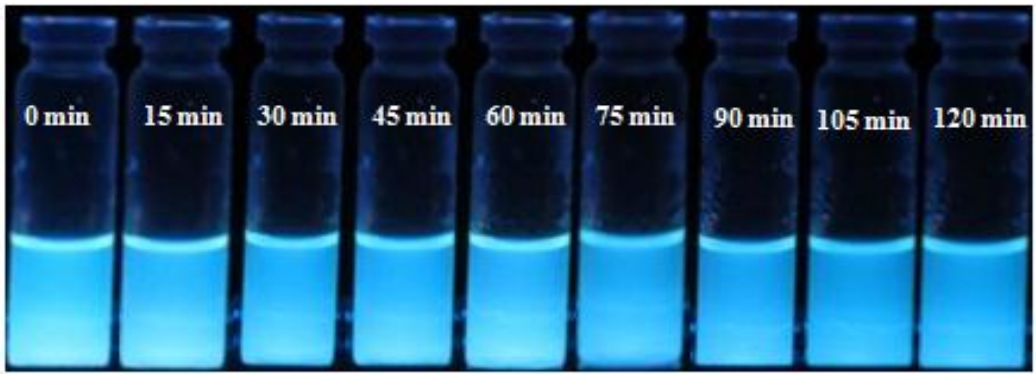


Figure 5



Under UV-light at λ_{ex} 365 nm

Figure 6

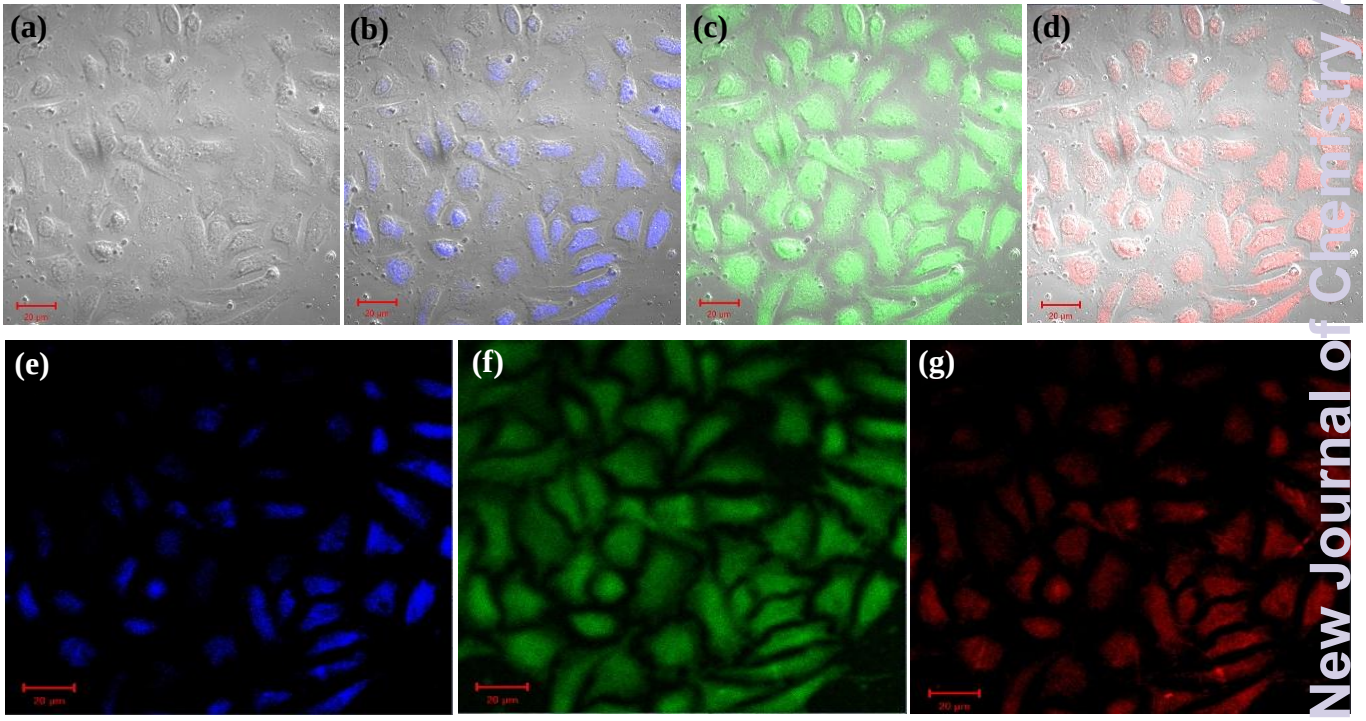


Figure 7

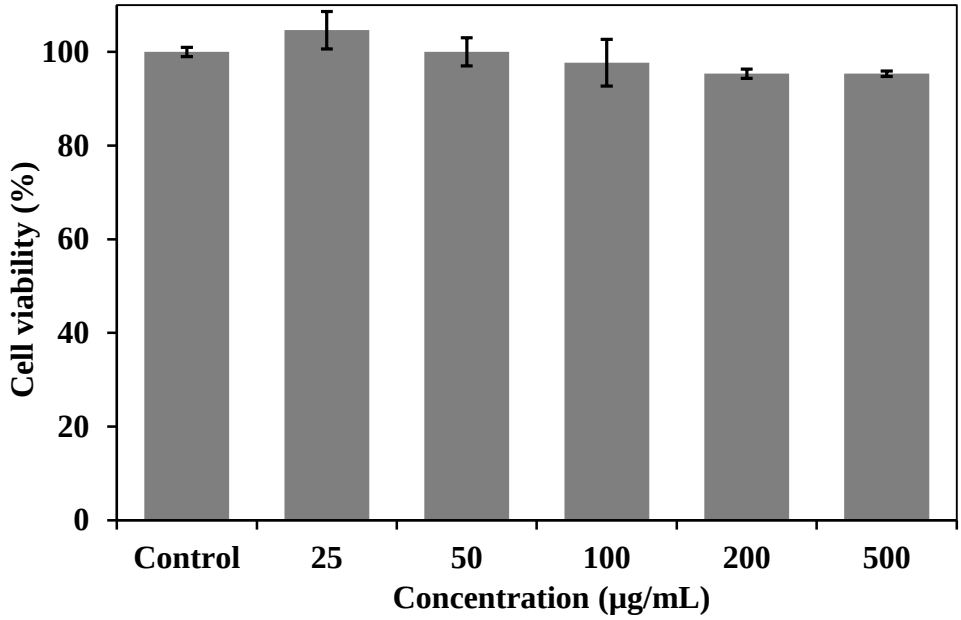


Figure 8