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Carbon Quantum Dots for Bioimaging

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Abstract

Carbon is nature's most abundant and useful element. Carbon nanomaterials with small size and unique optical properties have attracted tremendous interest for their promising biomedical applications. Carbon nanoparticles that exhibit fluorescence property are called as carbon quantum dots and they have emerged as a new class of carbon-based nanomaterials. In this chapter, we look at the unique properties of carbon quantum dots, their synthesis, material as well as optical characterizations, origin of fluorescence nature and applications of carbon quantum dots in bioimaging.

Keywords: carbon quantum dots, bioimaging, nontoxic fluorogens, fluorescence, optical imaging and green synthesis

1. Introduction

Fluorescent nanodots have drawn much attention in the twenty-first century for a wide range of applications specifically in biomedical field such as bioimaging, biosensing, drug delivery and photodynamic therapy. In the midst of various biomedical fields, bioimaging is one of the most powerful tools for live cell imaging studies via fluorescence microscopy. It endows with the direct visualization of processes occurring in biological species. Copious reports are available for the usage of organic and inorganic nanomaterials as fluorophores toward bioimaging applications.

In bioimaging studies, it is necessary to use a fluorescent probe to view the image of the cells/specimen species. Numerous organic fluorophores are commercially available which exhibits high-emission quantum yield (ϕ). The better one is Rhodamine 6G, which shows the ϕ value of 80%. But there are few laggings in the usage of organic fluorophores in bioimaging: they are prone to photobleaching and are easy to drip out from the cytoplasm to media, have

less photostability (i.e., the fluorophore will undergo degradation while irradiating with light during the imaging process), narrow excitation and emission wavelength (no possibility of tuning the optical properties) and cytotoxicity (during the bioimaging process, organic fluorophore may change the metabolism of live cells), etc.

Compared to organic fluorophores, metal-based chalcogenide inorganic semiconductor nanocrystals such as quantum dots (QDs) like CdX (X = Se, Te) capped with various long-chain alkyl thiols were extensively used as fluorescent probe during the last decade. The advantages of semiconductor QDs over organic fluorophores are due to their unique property such as quantum confinement effect (i.e., change in particle of QDs leads to changes in optical properties, for example, increase in particle size will shift the absorption in the longer wavelength due to the reduction in bandgap energy), one can vary the optical properties (absorption, emission wavelengths and emission quantum yield) simply by tuning the particle size during the synthesis process or postsynthetic treatments. Moreover, quantum dots show high photostability compared to organic fluorophores since they have the core-shell structure capped with ligands. At the same time, their drawback is in view of biocompatibility, since most of the QDs exist with the presence of toxic heavy metals like Cd, Pb and Hg in their composition. Even though they were enfolded with the surface coverage, within the cell during the imaging analysis, there is a chance of release of those toxic metals into the medium that are harmful to the live cells.

So, in order to conquer the abovementioned problems of unfortunate biodegradability and toxicity, novel fluorescent materials with better optical and biological properties should be developed for bioimaging of live cells. In this context, carbon quantum dots (CQDs), one of the present century invented member of nanocarbon family (**Figure 1**), have emerged as potential candidates for application in such essential field. Compared to the conventional organic fluorophores and recent inorganic semiconductor QDs, the newly emerged carbon

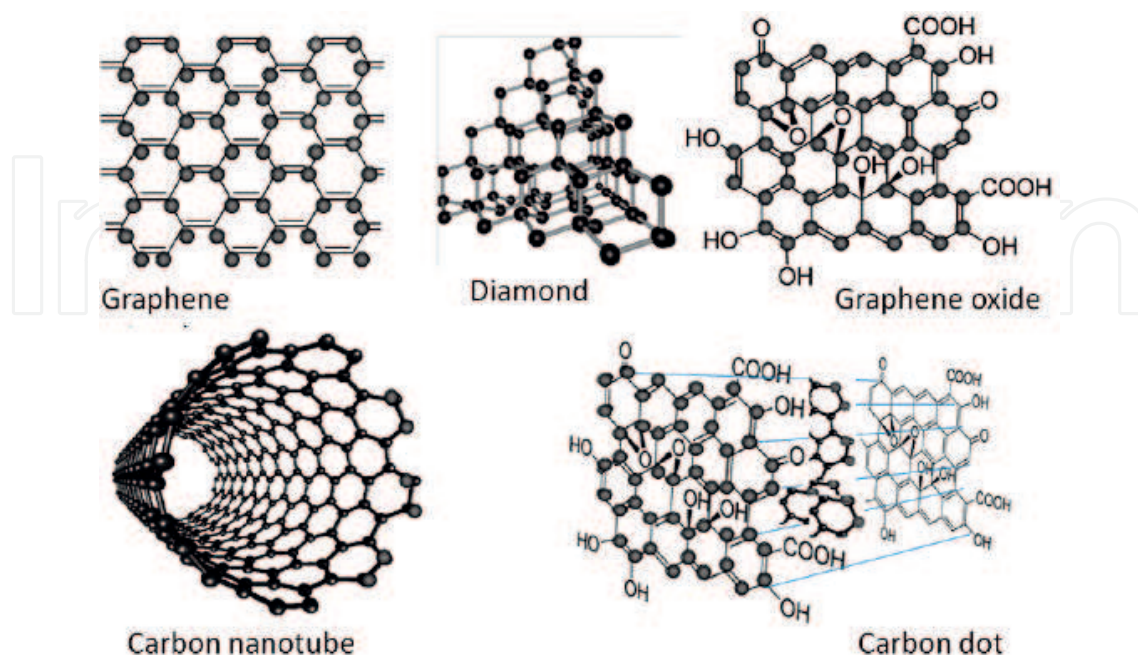


Figure 1. Structures of nanocarbon family members (reproduced with permission from [2]).

quantum dots are superior in terms of resistance to photobleaching, chemical inertness, facile surface functionalization, etc. Interestingly, carbon quantum dots are exhibiting low cytotoxicity, high aqueous solubility and are rich in emission quantum yield that entrusts them for the utilization in the field of biomedical research especially in *in vivo* and *in vitro* bioimaging.

The discovery of fluorescent carbon nanoparticles was accidental, during the separation and purification of single-walled carbon nanotubes via electrophoresis by Xu in the year of 2004 [1]. After 2 years, Sun and his research group named the small carbon nanoparticles as carbon quantum dots derived from graphite powder using laser ablation technique. In these 12 years, carbon quantum dots have attracted most of the chemists as well as biologists owing to their distinct properties such as abundance in nature, inexpensiveness, facile synthesis, high surface area, flexible functionalization, nontoxicity, photostability, high emission quantum yield, high water soluble nature, etc. This chapter focuses on the synthesis, functionalization, electronic structure, origin of fluorescence property and process involved in the application of carbon quantum dots in bioimaging.

2. Synthesis of carbon quantum dots

During the last 10 years, there were numerous methods reported for the synthesis of carbon quantum dots, which can be broadly classified into two types such as top-down and bottom-up approaches. In both the types, one needs to tackle the problems such as aggregation during the carbonization process, homogeneity as well as particle size control and surface properties. In order to overcome these obstacles, there are solutions given like adopting perfect synthetic routes and postsynthetic treatments. Carbon quantum dots can be synthesized from various precursors such as chemical, green and waste materials. In this section, few effective synthetic methods in view of simple, large-scale production and economical were discussed.

2.1. Top-down synthetic routes

2.1.1. Electrochemical

This technique is based on the electrochemical carbonization of low-molecular weight organic compounds by applying direct current. Electrochemical workstation instrument can be used for this method. In this process, low-molecular weight organic compounds like alcohols undergo electrochemical carbonization reaction under basic conditions. Three-electrode system contains two Pt sheets as the working and counter electrodes, as well as a calomel electrode mounted on a freely adjustable Luggin capillary acted as the reference electrode; these three electrodes were fixed with a rubber plug, and the distance between the two Pt sheets to be about 3 cm. The precursor solution is prepared by mixing alcohol with water in the ratio 14:1 in a basic medium by adding sodium hydroxide under stirring. The duration of reaction is about 4 h at a suitable potential until the transparent solution turns dark brown in color. The current density can be varied from 15 to 100 mA cm² depending on the precursor molecules selected. Once the reaction is completed, equal volume of ethanol is added in the reaction mixture to salt out the NaOH, and the mixture is left overnight. Further evaporation

of solvent, the solid product can be derived that needs separation and purification for the removal of unreacted small molecules by dialysis against water using membrane to obtain the carbon quantum dots. **Figure 2** shows the overview of preparation.

Advantages of this method are as follows: amorphous carbon quantum dots can be obtained by this technique that imparts high fluorescence quantum yield (maximum of 16%). Quantum confinement effect can be achieved by this technique such as size-dependent photoluminescent properties simply by tuning the applied current density. Purification of the product obtained is facile, and easy surface passivation is possible. At the same time, there are few limitations such as this method is economically costlier for large-scale production; surface defects cannot be avoided and production yield will be lower.

2.1.2. Laser irradiation

In this process, a carbon target in the presence of water vapor with an inert gas as a carrier under high temperature and pressure is irradiated with laser beam. In a typical procedure, carbon precursor is dispersed in some solvent by ultrasonication, and the suspension is dropped into a glass cell for laser irradiation. Generally, Nd:YAG pulsed laser with a second harmonic wavelength of ~ 530 nm is used to irradiate the suspension. After laser irradiation for different times, the reaction mixture undergoes centrifugation, purification and surface passivation processes to obtain fluorescent carbon quantum dots. **Figure 3** shows the process involved in laser irradiation technique. Advantages of the laser irradiation technique are that it is a fast synthetic route and environmentally friendly approach for the synthesis of carbon quantum dots, but this method is economically not favorable, skilled personal is necessary to conduct the synthesis and postsynthetic process such as surface passivation is necessary to impart fluorescent nature.

2.2. Bottom-up synthetic routes

2.2.1. Microwave pyrolysis

This method involves the microwave irradiation of organic compounds in presence of reaction medium. This is a rapid and low-cost method to prepare carbon quantum dots, and in general, sugar moieties were used as a carbon source and polymeric oligomers as reaction media

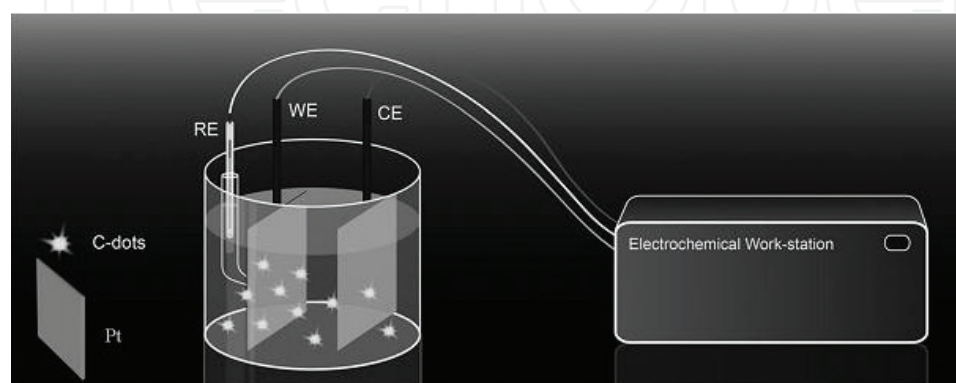


Figure 2. Electrochemical synthesis of C-dots. Under basic conditions (reproduced with permission from [3]).

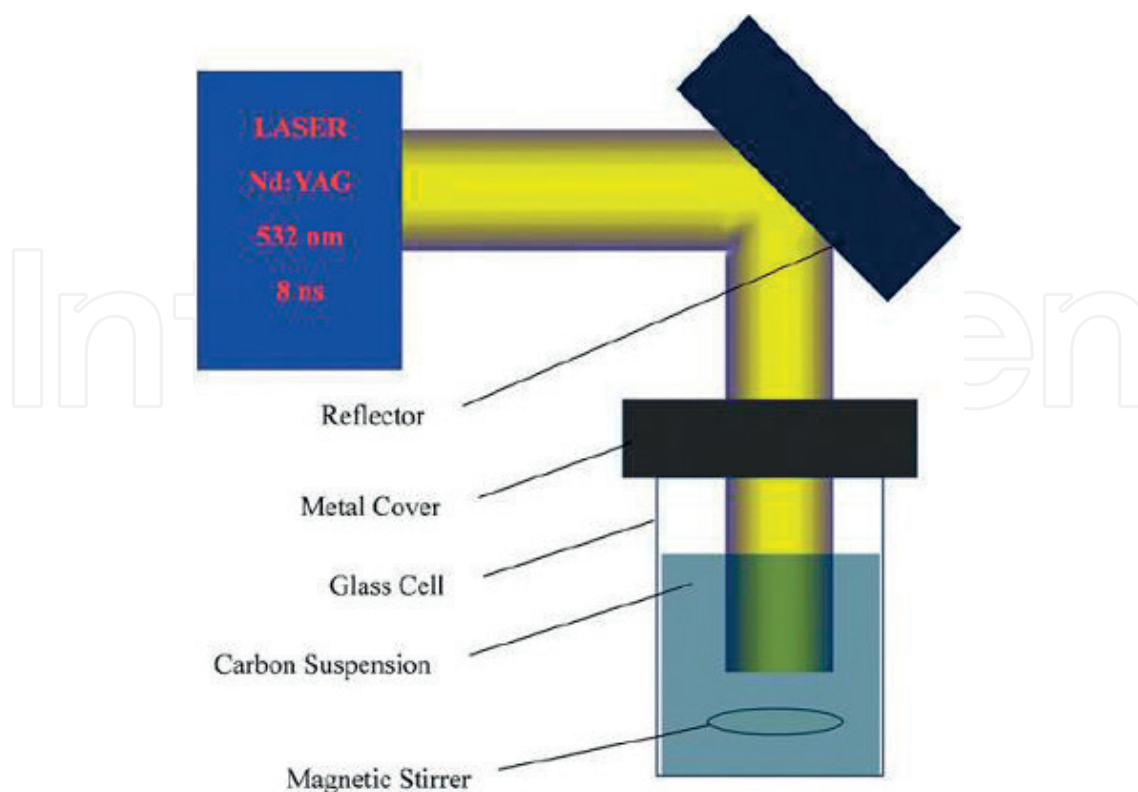


Figure 3. Schematic illustration of experimental setup for the synthesis of carbon quantum dots by laser irradiation technique (reproduced with permission from [4]).

and amine molecules play a role of nitrogen dopants and surface-passivating agents in order to improve the emission quantum yields. Initially, different amounts of reaction medium and carbon precursor are mixed with distilled water. Further, the resultant transparent solution is heated in a microwave oven of 500 W for 2–10 min. Within a minute, color changes from colorless to pale yellow, and further, it increases to dark brown indicating the formation of carbon quantum dots. After cooled to room temperature, the product undergoes separation and purification processes to obtain fluorescent carbon quantum dots (**Figure 4**). Advantages of microwave pyrolysis route are as follows: simple, rapid and efficient method for the synthesis of carbon quantum dots [5]. At the same time, there are few limitations: being costly and the presence of harmful microwave radiation, which is to be handled with precautions.

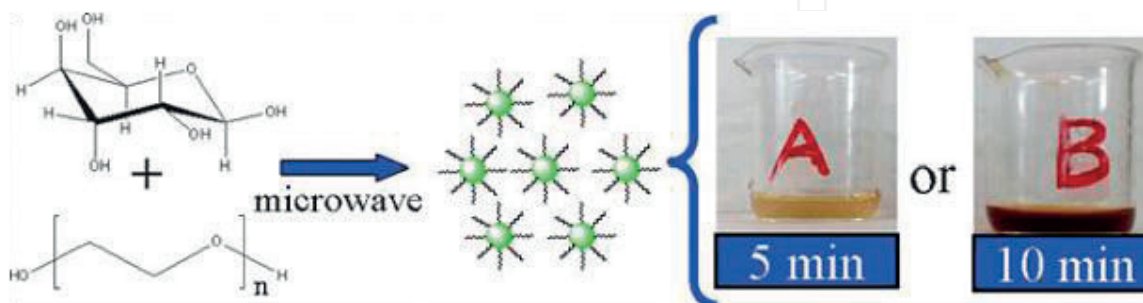


Figure 4. Microwave pyrolysis approach for the synthesis of carbon quantum dots (reproduced with permission from [5]).

2.2.2. Oxidative acid treatment

This treatment includes the reflux of waste soot in acidic medium followed by centrifugation, neutralization and purification via dialysis against water. Typically, a few gram of waste soot collected by combustion of either natural gas or any other fuel material is mixed with 5 M nitric acid and refluxed for 12 h. After cooled down to room temperature, fluorescent carbon particles were collected by centrifugation. Alternatively, the mixture was first neutralized by Na_2CO_3 , and then extensively dialyzed against double distilled water through dialysis membrane (MWCO 1000). **Figure 5** shows the images of carbon soot and carbon quantum dots derived after the oxidative acid treatment process. Advantages of this acid treatment are effective to introduce functional groups such as carbonyl, carboxyl, amines and epoxy, etc., toward greater water-soluble nature of carbon quantum dots, postsynthetic aggregation can be avoided, and without surface passivation, the prepared carbon quantum dots can be used for imaging applications. A disadvantage of this treatment process is defects may reduce the storage stability of prepared carbon quantum dots.

2.2.3. Hydrothermal/solvothermal synthesis

This is the most popular and facile method for the synthesis of fluorescent carbon quantum dots in which various sources such as chemical as well as green precursor can be used for the synthesis of carbon quantum dots. This technique involves a solution of organic precursor in the presence of either water or some organic solvent sealed in an autoclave (reactor setup which is made up of stainless steel outer and Teflon-coated inner lining to withstand high temperature and pressure, **Figure 6**) and reacted at high temperature (less than the critical temperature of the solvent taken). After complete carbonization, it is autoclaved to allow cooling down to room temperature naturally, and the products can be extracted with an organic

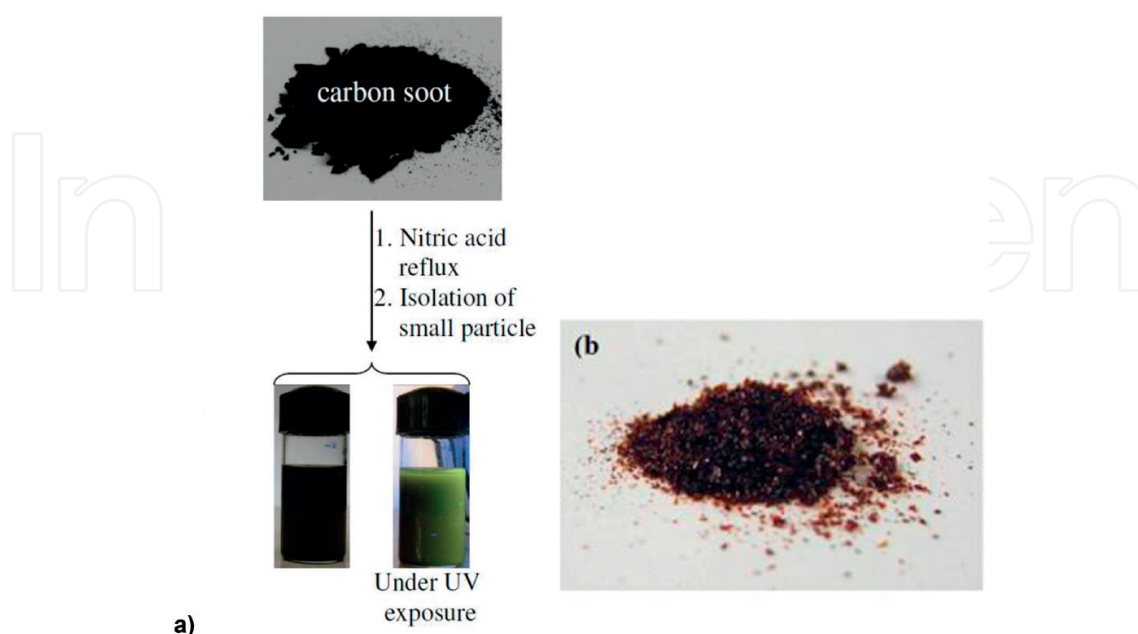


Figure 5. Oxidative acid treatment for the synthesis of green fluorescent carbon quantum dots and image of the functionalized carbon quantum dots. (reproduced with permission from [6]).



Figure 6. Synthesis of blue emissive carbon quantum dots from gelatin via hydrothermal carbonization process (reproduced with permission from [7]).

solvent. By adopting this technique, one can synthesis both hydrophilic and hydrophobic fluorescent carbon quantum dots simply by selecting the appropriate precursors.

Compared with other routes, the hydrothermal carbonization process has some of the advantages such as low toxicological impact of materials and processes, the use of renewable resources, facile instrumentation techniques and high atom economy. This method is cost-effective, ecofriendly and nontoxic. Using this technique, large-scale production is possible and reaction conditions such as time and temperature are adjustable. Postsynthetic surface passivation is not necessary. The only limitation of this method is poor control over particle sizes [7].

2.3. Sources of precursors

Carbon quantum dots can be synthesized from wide variety of precursors like both chemical and green resources. **Table 1** shows the range of precursor materials reported for the synthesis of carbon quantum dots especially for bioimaging application with the value of emission quantum yield.

Precursor	Synthetic method	Quantum yield (ϕ)	Reference [8]
Glucose	Hydrothermal treatment at 200°C	1.1–2.4	55
Candle soot	HNO ₃ oxidation	3	57
Ascorbic acid	Hydrothermal treatment at 140°C	5.7	53
Sugarcane juice	Hydrothermal treatment at 120°C	5.76	62
Watermelon peels	Carbonization at 220°C	7.1	58
Hair fiber	H ₂ SO ₄	11.1	68
Phenol/formaldehyde resin, silica particle	Carbonization at 900°C, NaOH etching	14.7	3
Orange juice	Hydrothermal treatment at 120°C	26	60
Gelatin	Hydrothermal treatment at 200°C	31.6	67
Chitosan	Hydrothermal treatment at 180°C	43	64

Table 1. Carbon quantum dots synthesized by green route for imaging applications [8].

2.4. Surface functionalization and passivation

Modification of the surface of carbon quantum dots is necessary for selective application like bio-imaging which can be achieved by either surface passivation or functionalization (Figure 7). The former one reduces the detrimental effect of surface contamination to their optical properties and to impart high fluorescent intensity. This can be done by formation of a thin insulating layer of coating materials such as oligomers (poly ethylene glycol (PEG)), thionyl chloride, thiols and spiropyran, etc., on the surface of carbon quantum dots. The process of acid treatment and surface passivation enhances the quantum yield of carbon quantum dots to the maximum of 55–60% which can be attained by the soft shell of passivation agents that surround the hard fluorescent core of the carbon quantum dots. Thus, one can achieve the improved fluorescence emissions.

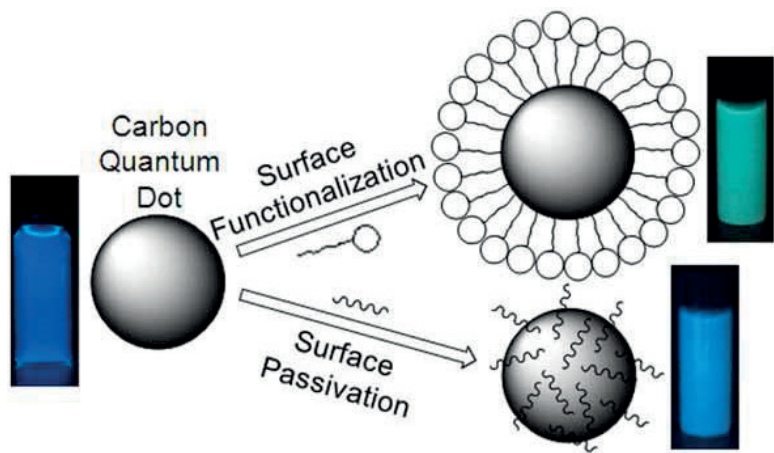


Figure 7. Graphical representation of surface passivation and functionalization of carbon quantum dots (reproduced with permission from [9]).

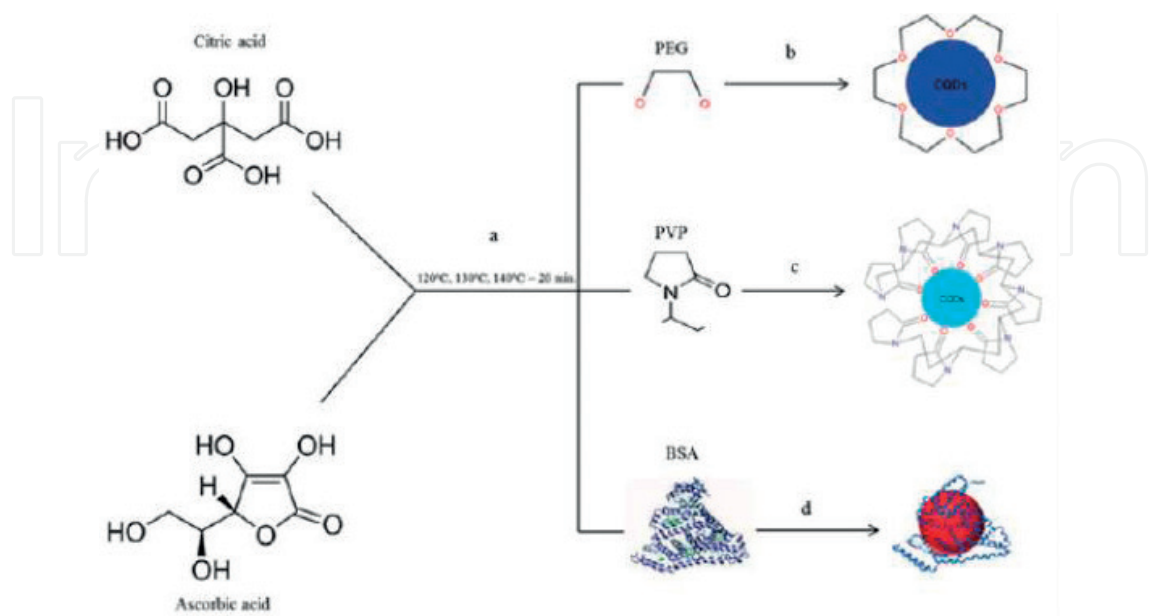


Figure 8. Carbon quantum dots synthesized from citric acid and ascorbic acid functionalized with (a) polyethylene glycol, (b) polyvinylpyrrolidone and (c) bovine serum albumin (reproduced with permission from [10]).

The later process such as surface functionalization (**Figure 8**) is important due to the introduction of functional groups like, carbonyl, carboxyl and amines which can impose various surface defects on carbon quantum dots. These defects can act as surface energy traps and lead to variations in fluorescence emission behavior of carbon quantum dots. Surface functionalization can be attained by surface chemistry or interactions like coordination, π - π interactions and covalent bonding, etc. Since the carbon quantum dots are oxygenous in nature, they are feasible to covalent bonding with functionalizing agents. Compared to bare carbon quantum dots, the functionalized one are excellent in photoreversibility, high stability, good biocompatibility and low toxicity.

Sometimes, few compounds may use as both passivating and functionalizing agent in which there is no need of additional modification steps during the postsynthetic treatments [11]. High emission quantum yield is essential for carbon quantum dots in order to overcome their counterparts such as organic dye molecules and inorganic semiconductor quantum dots. Other than surface passivation and functionalization, one can use the doping of carbon quantum dots with heteroatoms and nitrogen which can enhance the quantum yield up to 83%.

3. Characterization techniques

This section introduces some commonly used sophisticated techniques for the characterization of carbon quantum dots.

3.1. Microscopes

These are highly useful to analyze the surface morphology, particle size, structure and even more composition of carbon quantum dots (**Figure 9**). Few powerful microscopes such as high-resolution scanning electron microscope (HR-SEM), transmission electron microscope (TEM) and atomic force microscope (AFM) are able to resolve the structure of carbon quantum dots up to atomic resolution. The TEM specimen can be prepared by depositing a few drops of a diluted carbon dot solution onto a carbon-coated copper grid, followed by complete evaporation of the water solvent. Diameter of carbon quantum dots can be calculated by using Image J software resulting in the area of particles present: $A = \pi r^2$, $r = \sqrt{(A/\pi)}$ and $D = 2 \times r$. In this, A is the area of particles occupied, r is the radius of particles and D is the diameter of the particles [12]. The AFM specimen on a mica surface is usually prepared as similar to TEM analysis and both tapping and noncontact modes can be used for the AFM image measurement. SEM measurement is usually done for powder sample.

3.2. Diffraction techniques

Diffraction techniques are mostly used for structural determination (amorphous/crystalline) and average particle size analysis (crystallite size). Powder X-ray diffraction (XRD) and small-angle X-ray scattering (SAXS) are the commonly used techniques.

3.3. Spectroscopic techniques

Wide range of spectroscopic techniques can be used for the analysis of optical properties of carbon quantum dots. These techniques are useful for chemical state analysis (bonding or

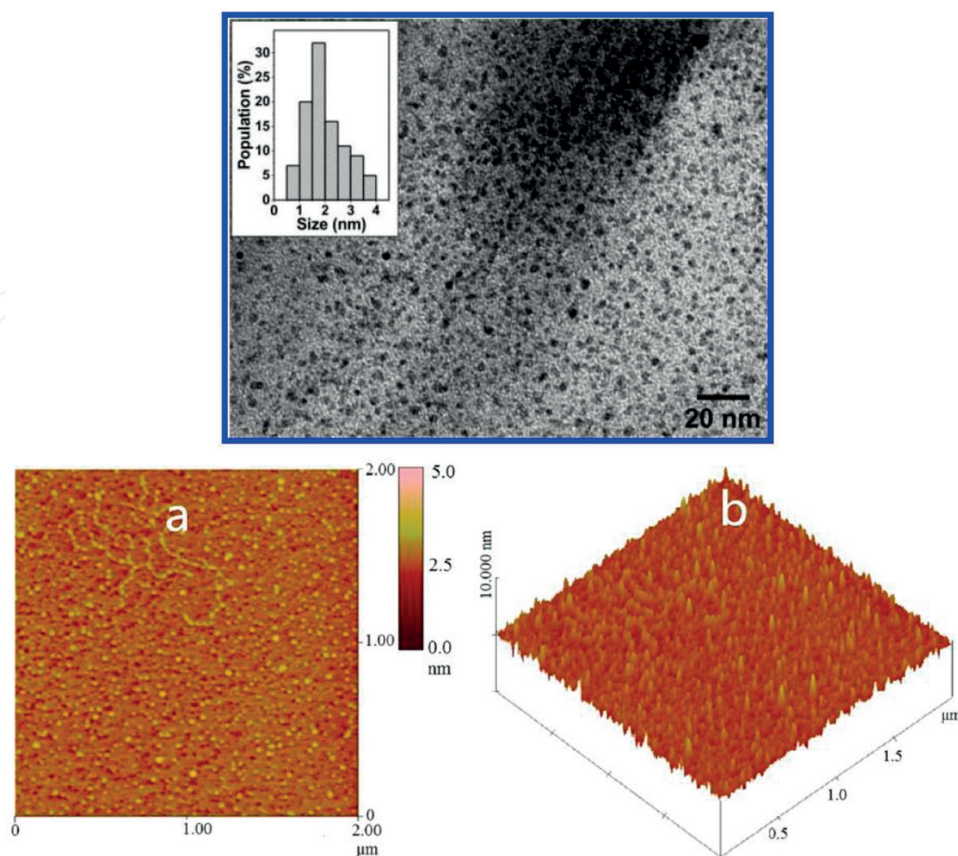


Figure 9. High-resolution TEM and AFM images of carbon quantum dots (reproduced with permission from [13]).

charge transfer among the atoms) and electronic structure (bandgap energy, level of impurity, band formation and possibilities of electronic transitions, etc.). This includes UV-visible-near IR absorption (both transmission and reflection mode), Fourier transform infrared (FT-IR), atomic absorption spectroscopy (AAS), nuclear magnetic resonance (NMR, both ^1H and ^{13}C NMR), Raman spectroscopy, X-ray photoelectron spectroscopy (XPS), Auger electron spectroscopy (AES) and electron spectroscopy (both spin and resonance, ESR and EPR) (**Figure 10**).

Carbon quantum dots typically show two optical absorption band (**Figure 11**) in the UV region with extended tail in the visible range. The two absorption shoulders are attributed to the π - π^* transitions in C=C bonds and n- π^* transition of C=O and other oxygenous carbon-containing functional groups.

3.4. Other techniques

Other than the abovementioned techniques, few techniques such as elemental analysis, zeta potential analyzer, dynamic light scattering analysis, fluorescence spectroscopy and time-correlated single photon counting (TCSPC) techniques are useful for the study of percentage of elemental composition, surface charge/dispersivity nature, hydrodynamic particle size (average particle size in solution medium), emission nature (wavelength of emission as well as emission quantum yield) and excited-state lifetime of carbon quantum dots, respectively.

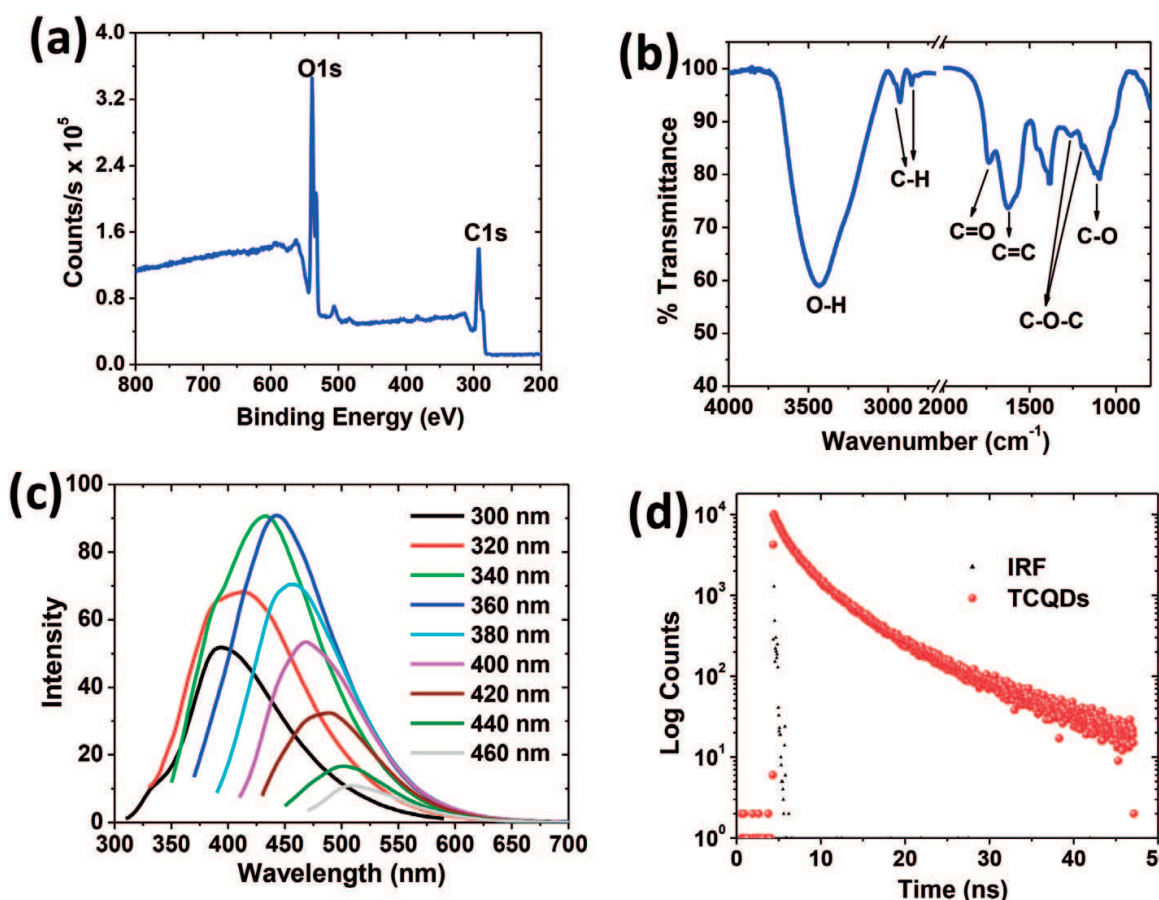


Figure 10. (a) XPS survey spectrum, (b) FTIR spectrum, (c) excitation-dependent emission spectra and (d) fluorescence decay of carbon dots prepared from tamarind (reproduced with permission from [14]).

3.5. Structure of carbon quantum dots

Fluorescent nanodots can be classified into four types (**Figure 12**) such as semiconductor quantum dots (SQDs), graphene quantum dots (GQDs), carbon quantum dots (CQDs) and carbon nanodots (CNDs).

SQDs are nanometer scale semiconductor crystals composed of group II metal ions as building blocks, and are defined as particles with physical dimensions smaller than the exciton Bohr radius (~ 7 nm). The excitons in these particles are confined in the spatial dimensions with quantized energy states. The best examples for SQDs are chalcogenide-based CdX QDs ($X = \text{S}, \text{Se}, \text{Te}$).

CNDs are amorphous quasispherical nanodots that lack in quantum confinement, and when the nanodots present as π -conjugated single sheet, i.e., a disk of graphene sheet in the size range of 2–10 nm, they are called GQDs.

CQDs are typically quasispherical nanoparticles comprising amorphous to nanocrystalline cores with predominantly graphitic or turbostratic carbon (sp^2 carbon) or graphene and graphene oxide sheets fused by diamond-like sp^3 hybridized carbon insertions. CQDs exhibit

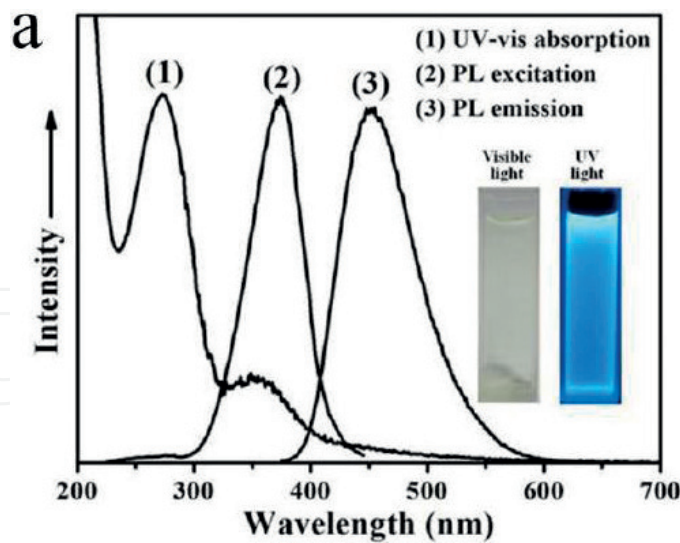


Figure 11. Absorption, excitation and emission bands of carbon quantum dots (reproduced with permission from [15]).

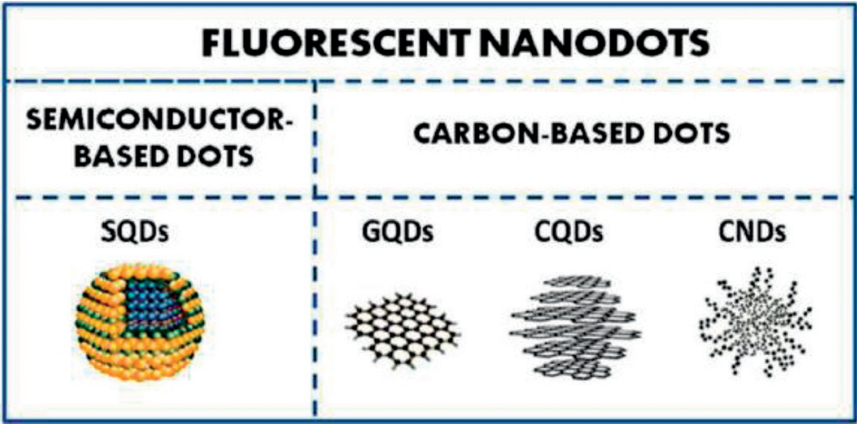


Figure 12. Classification of fluorescent nanodots: semiconductor quantum dots (SQDs), graphene quantum dots (GQDs), carbon quantum dots (CQDs) and carbon nanodots (CNDs) (reproduced with permission from [16]).

quantum confinement effect (size-dependent optical properties). Depending on the synthetic route, the oxygen content in the oxidized CQDs ranges from 5 to 50% (weight). Figure shows the difference among the various fluorescent nanodots with their plausible structure.

3.6. Fluorescent properties of carbon quantum dots

The exact origins of the fluorescence emission nature of carbon quantum dots remain debatable, and lot of research is ongoing to draw a clear picture of the fluorescence mechanism. For the two types of emissions observed for carbon quantum dots such as excitation dependent/ excitation independent, two classes of mechanism have been proposed (Figure 13). The first class is bandgap transitions caused by π -domains, and the second class is associated with surface defects on carbon quantum dots [17].

The bandgap electronic transitions display strong absorption in the ultraviolet region, but weak emission and the surface defect-derived origin exhibits weak absorption in the near-UV

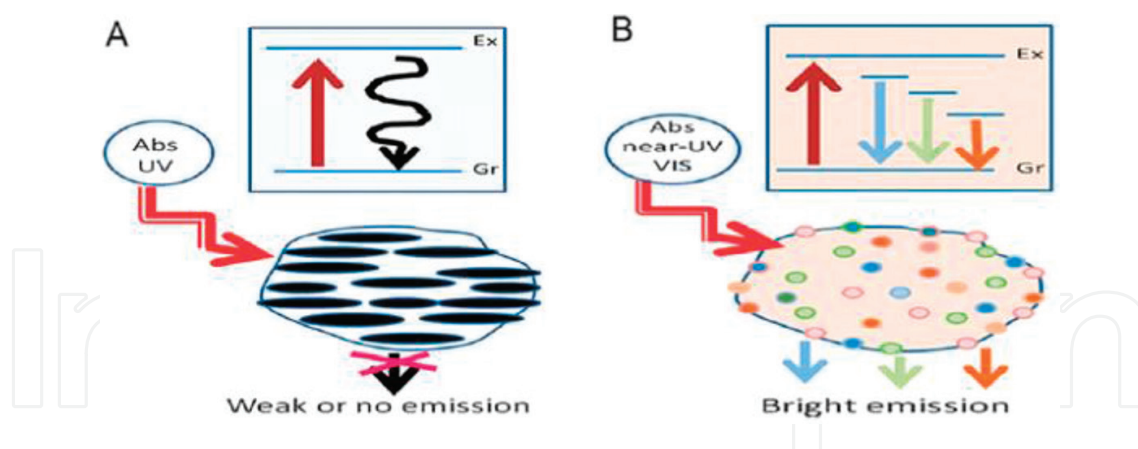


Figure 13. (a) Bandgap transitions caused by π - π domains and (b) transition caused by surface defects on carbon quantum dots (reproduced with permission from [18]).

and strong emission in the visible region [18]. In addition, due to surface passivation/functionalization, the defect becomes more stable to facilitate more effective radiative recombination of surface-confined electron and hole leads to more bright emissions.

The quantum yield (ϕ) of carbon dots can be calculated by using quinine sulfate as a reference compound in which ϕ value is reported as 0.53 [20]. For the measurement of ϕ , the optical density of tamarind carbon dots (TCDs) in water ($\eta = 1.33$) was fixed to less than 0.1 at the wavelength of 360 nm. Quinine sulfate is dissolved in 0.1 M H_2SO_4 ($\eta = 1.33$). For both the sample and reference, emission spectra were recorded at the same excitation at 360 nm. Fluorescence quantum yield (ϕ_F) for TCDs was calculated by using Eq. (1)

$$\phi_F = (A_R/A_S) (I_S/I_R) (\eta_S/\eta_R)^2 \phi_R \quad (1)$$

where the subscript 'S' refers to the samples, the subscript 'R' refers to quinine sulfate, A is the absorbance at the excitation wavelength, I is the integrated emission area and η is the solvent refraction index.

3.6.1. Enhanced fluorescent properties of carbon quantum dots by doping

Carbon quantum dots with enhanced emission quantum yield are necessary for better imaging of cells. Other than surface passivation, doping and codoping of heteroatoms such as nitrogen and sulfur imparts high quantum efficiency. Recently, it is reported that about 80–83% of quantum yield can be achieved by doping of carbon quantum dots with nitrogen and Mg ion. Doping with metal ions also improves the optical properties in addition with creation of novel functionalities. But one has to concern about the chance of increase in toxicity by doping with metal ion. Compared to metal ion doping, heteroatoms show better advantages due to their atomic size comparable with carbon quantum dots.

Owing to the close resemblance between nitrogen and carbon, the former one is preferentially used as a dopant for carbon quantum dots. Here, nitrogen can donate its electrons to carbon quantum dots, which change the electronic configuration leading to improvement in quantum yield. Nitrogen can be incorporated with carbon quantum dots either during the synthesis

via adding nitrogen-containing precursors or postsynthetic functionalization by agents built with nitrogen atom such as amine derivatives. Sulfur, phosphorous and boron atoms have also been as dopants in view of enhancing the fluorescence quantum yield of carbon quantum dots. Codoping of multiple heteroatoms in carbon quantum dots has gained more attention because of the synergistic effect between codoped heteroatoms and carbon quantum dots creating unique electronic structure [19].

4. Application of carbon quantum dots: bioimaging

Live cell bioimaging is becoming an increasingly popular tool for elucidation of biological mechanisms and is instrumental in unraveling the dynamics and functions of many cellular processes. Bioimaging is a method for direct visualization of biological processes in real time which often used to gain information on the 3D structure of the observed specimen from the outside, i.e., without physical interference. Bioimaging spans the observation of subcellular structures using light, fluorescence, electrons, ultrasound, X-ray, magnetic resonance and positrons as sources for imaging. It can be used to follow cellular processes, quantify ion or metabolite levels and measure interactions of molecules live. Appropriate tracers, e.g., specific fluorochromes, and advanced microscopic instruments, e.g., confocal laser scanning microscopes (CLSM, **Figure 14**), are a prerequisite for most applications.

4.1. Basic requirements for successful imaging

Imaging of biological processes in cells is highly dependent on the conditions provided. In view of avoiding the cellular stress, the environmental conditions should be nearly close to natural cellular ambience. Depending on the type of cell, the medium has to be changed. Generally, Dulbecco's modification of MEM (DMEM) and Roswell Park Memorial Institute (RPMI) are used as medium in which serum is added to provide all the essential nutrients for cell growth. Most of the mammalian cells grow between the pH 7.2–7.4. In bicarbonate-based medium, buffering is made by CO_2 whose withdrawal leads to cell growth. Considering

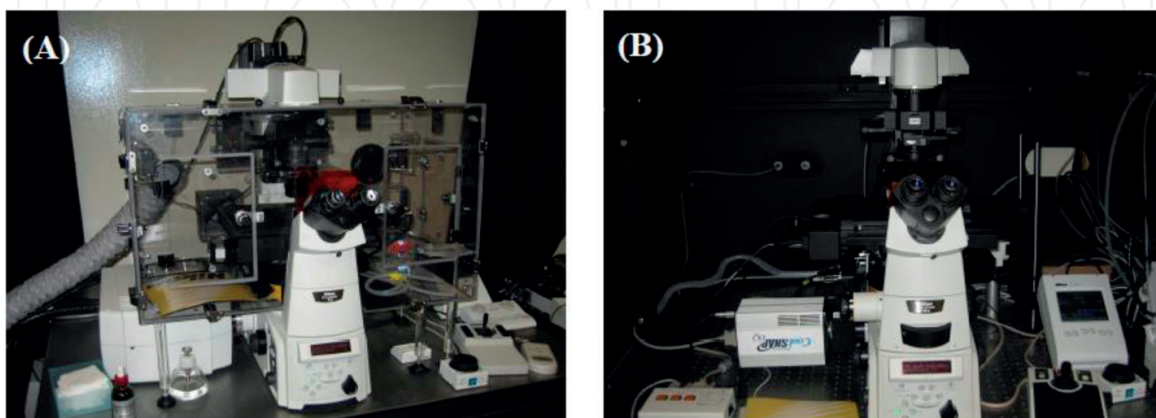


Figure 14. Bioimaging equipment with confocal facility (reproduced with permission from [21]).

cellular toxicity, compared to RPMI, DMEM is more prone to pH changes. So in view of minimizing the pH changes, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), a zwitterionic organic chemical, buffering agent is generally used in imaging studies.

It is also necessary to maintain a physiological temperature to avoid the cellular detachment or changes in morphology during prolonged exposure of cells. Generally, 37°C is maintained by temperature regulatory system to avoid the rapid evaporation of the medium during prolonged imaging process. Phototoxicity and photobleaching are the two common challenges of live cell imaging. For instance, changes in membrane structure, cell death, vacuolation and blebbing are the possible effect of high-energy light radiation such as laser or ultraviolet light as a light source. This is because when the light radiation interacts with the cells, the rise in temperature due to excitation of light active molecules may stimulate the formation of free radicals (which may attack cellular membrane, lipids, etc.) and laser light may trigger heat stress (oxidative stress). So in order to reduce the oxidative stress and extreme changes in the temperature of medium, it is necessary to use minimum energy/low-intense light radiation source (**Figure 15**).

Loss of fluorescent signals during overtime of live cell imaging is called photobleaching. Photosensitivity of fluorescent dyes, the expression level of fluorescent proteins and size of imaging objects are the factors influencing the photobleaching process. Live cell imaging of small fluorescent vesicles is more susceptible to photobleaching compared to the image acquisition of larger fluorescent organelles like nucleus.

4.2. Role of carbon quantum dots in successful bioimaging

As discussed in the above section, the factors affecting the live cell bioimaging are cell viability, efficiency as well as resolution of confocal microscope and nature of the fluorescent component used. The last one such as a fluorescent contrast agent, which should possess biocompatibility and low biocytotoxicity, is necessary for straightforward fluorescence imaging of live cells and tissues. Depending on the encapsulation/functionalization, carbon quantum dots are either localized in the cell membrane or cytoplasm. Distribution of carbon quantum dots throughout the cytoplasm is also important for the high resolution of the image.

It is necessary to check the cytotoxic nature of carbon quantum dots before using it as a fluorescent contrast agent in bioimaging. For that, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay is generally followed in which the type of interest cells

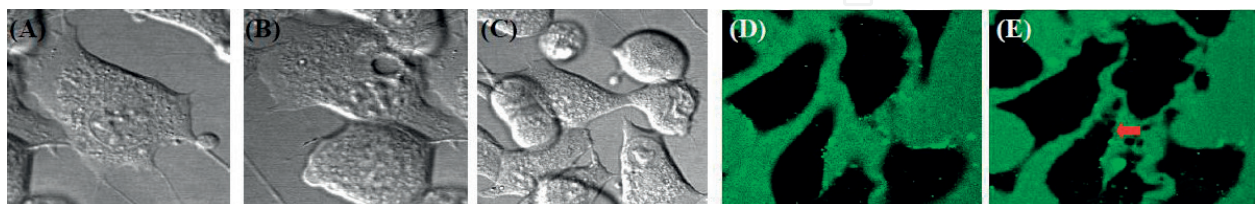


Figure 15. Morphological changes in BHK cells due to phototoxicity such as (A) spindle-shaped membranes, (B) vacuolation and (C) rounding of cells. As shown in (D), the BHK cells displayed normal cell shape before imaging but prolonged exposure to activation laser caused membrane blebbing (indicated by arrow) and cell death (E) (reproduced with permission from [21]).

was harvested and the cell concentration was adjusted to 1×10^4 cells/ml; cells were placed in a 96-well flat-bottom culture plates (180 μ l/well) and incubated with various concentrations (0.78, 3.185, 12.5, 25.0, 50.0, 100.0 and 200.0 μ l/ml) of carbon quantum dots (in 20 μ l). All cultures were incubated for 72 h at 37°C and 5% CO₂ in a humidified incubator. Viable cell concentration was checked by MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay.

Similarly, for the study of intracellular uptake, the cell line (maintained in Dulbecco's modified Eagle's medium or DMEM supplemented with 10% fetal bovine serum or FBS) was trypsinized and seeded in tissue culture plates at 3×10^4 cells/well. After overnight incubation inside humidified 5% CO₂ incubator for cell attachment, the cells were treated with the carbon quantum dots at a final concentration of 200 μ g/ml in 300 μ l of media and incubated for 12 h. Prior to the imaging experiment, the cells were washed three times with fresh media.

This internalization of carbon quantum dots by the cells is a temperature-dependent process that happens at the ambient temperature (37°C). In general, carbon quantum dots likely translocate into the cell by endocytosis (i.e., process in which a substance gains entry into a cell without passing through the cell membrane resulting in the formation of an intracellular vesicle by virtue of the invagination of the plasma membrane and membrane fusion). The uptake of carbon quantum dots can be enhanced by coupling of carbon quantum with few membrane translocating peptides, so that it can facilitate the translocation process by overcoming the cell membrane barrier.

The excitation-dependent emission behavior of carbon quantum dots allows one to control and choose the excitation and emission wavelength. Carbon quantum dots with or without surface

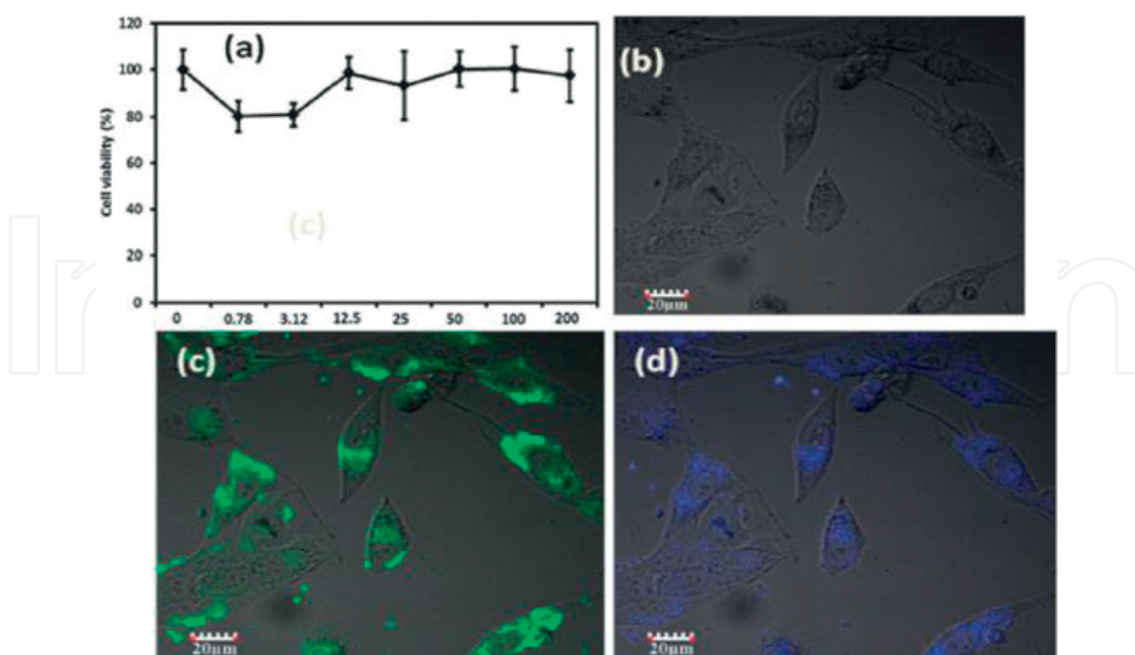


Figure 16. (a) Cell viability by MTT assay, (b) MG-63 cells under bright field, by excitation at (c) 488 nm and (d) 405 nm (reproduced with permission from [21]).

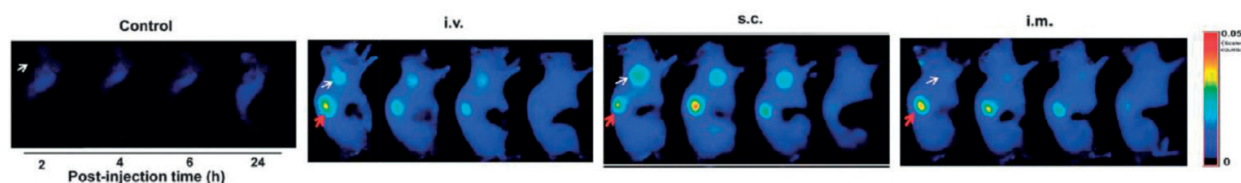


Figure 17. Fluorescence images of tumor-bearing mice (reproduced with permission from [21]).

passivation can be used as a labeling agent if it exhibits high emission quantum yield. The exact mechanism of carbon quantum dots uptake by cells remains to be elucidated, at the same time it is suggested that internalization with significant infiltration in to the cell nucleus. Carbon quantum dots can be used as fluorescent markers to probe various cellular organelles including lysosome/endosome, golgi body, mitochondria and endoplasmic reticulum (**Figure 16**). For *in vivo*, intravenous injection is used and the organs were harvested and sliced for imaging. Heart, liver, spleen, kidneys, lungs, brain and small intestine can be viewed (**Figure 17**). CQDs can be efficiently and rapidly excreted from the body after injection in different routes. Their blood clearance was quick—only 1 h postintravenous injection. The retention time is somewhat longer after subcutaneous and intramuscular injection. Finally, the imaging process is done under confocal microscope with laser excitation of 405 and 488 nm, and fluorescence was collected in blue and green region.

5. Concluding remarks

Since the discovery of carbon quantum dots in a decade back, they were used as fluorescent contrast agents for imaging of live cells. The defects present in carbon quantum dots play an important role in the fluorescence nature. They can be synthesized by various simple methods using variety of precursors. Unfortunately, the high emission quantum yield of carbon quantum dots still remains rare and is essential to use them in bioimaging application. Moreover, the well-defined composition and structure of carbon quantum dots are not explored well. The origin of fluorescence from carbon quantum dots is also still in debate which follows different mechanisms. Imaging of nucleus of cell using carbon quantum dots is not yet reported, and the sensitivity or selectivity of bioimaging needs to be improved. So, in future, it is necessary to prepare carbon quantum dots which will overcome the abovementioned unexplored properties, and deep research to be done for the use of bioconjugated carbon quantum dots as theranostics agents for targeted drug delivery and therapeutic applications.

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