Cellulose hydrogel is a novel carbon-source and doping-material-carrier to prepare fluorescent carbon dots for intracellular bioimaging

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Abstract

Carbon dots (CDs) is considered as a potential candidate for biological labeling due to its excellent biocompatibility, and element-doping was usually used to improve its labeling brightness. Thus, the carbon source with material-carried function will be of importance to produce the element-doped CDs. In present work, cellulose hydrogel was used both as the carbon source and the doping-material-carrier to obtain the N-doped CDs. The groups in so-obtained CDs were measured by means of ultraviolet-visible spectrophotometer (UV-Vis), Fourier infrared spectroscopy (FT-IR), and X-ray photoelectron spectroscopy. The microstructure of CDs was observed by means of high-resolution transmission electron microscope. The fluorescence quantum yield (QY) of the CDs was detected, and the labeling brightness on living cells was subsequently investigated. Finally, the influence of element-doping on the cytotoxicity was measured. The experimental results showed that Cellulose hydrogel is a nice carbon source and doping-material-carrier to produce fine N-doped CDs due to its distinct spatial network structure. The carried NaOH and urea in cellulose hydrogel significantly improved the QY of the CDs from 0.0542 ± 0.0030 to the maximum 0.1965 ± 0.0013 . For the same kind of CDs, the smaller the particle size is, the higher the QY value is. The CDs immobilized in the cytoplasm of the living cells, and contributed to a non-specific fluorescent labeling. The labeling brightness is both the QY value of the CDs and the uptake rate of the living cells dependent. For the same cell line, the QY of CDs and the labeling brightness of living cells are significantly linear correlated. The cytotoxicity of CDs is low enough for a long-time observation on living cells.

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Graphic abstract

- Cellulose hydrogel is a nice carbon source and doping-material-carrier to produce fine N-doped CDs due to its distinct spatial network structure.
- The carried NaOH and urea in cellulose hydrogel raw material significantly improved the fluorescence quantum yield (QY) of so-obtained CDs. For the same kind of CDs, the smaller the particle size is, the higher the QY value is.
- For the same cell line, the QY of CDs and the labeling brightness of living cells are significantly linear correlated.
- The cytotoxicity of CDs is low enough to run a long-time observation on living cells.

Keywords Carbon dots · Cellulose hydrogel · Quantum yield · Labeling brightness · Living cells

1 Introduction

Carbon quantum dots (CDs), a new zero-dimensional nanomaterial, has advantages on the stable photoluminescence, excellent biocompatibility, and low cost when compared to those traditional semiconductor quantum dots (CdS, CdSe, CdTe, et al.). Thus, CDs is regarded as a potential candidate for application in ion/molecule detection, biosensors, bio-imaging, especially in living cell imaging [1–3].

A good fluorescent probe in living cells imaging should be low toxicity, high stability, bright fluorescence, good water solubility, and sometimes specific binding. As a fluorescent probe, CDs was demonstrated that it can be internalized into the cells to mark the cytoplasm and the membrane [4]. Different types of cells have different responses on CDs labeling, thus, CDs was considered to distinguish cancer cells from those normal cells. For example, CDs combined with folic acid was produced to distinguish between cancer cells and normal cells [5]. Receptor-mediated endocytosis of CDs provides a more accurate and selective method for cancer diagnosis [6]. However, the labeling brightness of CDs needs to be further improved, and the specific labeling property of CDs needs to be developed [7].

Element-doping is an effective way to improve such labeling properties as brighter imaging and specific labeling because it can introduce helpful groups to CDs. Labeling brightness is a key index for a fluorescent probe. To improve the labeling brightness of CDs and thus enables it to be more effectively used, such methods as passivation and elements doping were tried to modify the fluorescent properties of CDs [8]. For example, Nitrogen (N) doping can effectively accelerate the rate of electron transfer in the molecule without significantly increasing the size of CDs [9], thus, N-doped CDs exhibits a brighter labeling image. Phosphorus is also a non-metallic heteroatom with high electron-donating ability. The new active sites provided by phosphorus doping can improve the electron transfer ability [10]. Specific labeling is another property to approach, and element-doping can provide groups such as $-NH_2$, $-COOH$, etc. to bonding the target antibody or nucleic acid aptamer [6].

For the doping-element-modified CDs, the doping dose, doping yield, and doping methods need to be considered besides the doping materials. Normally, the mixture of the doping material and the carbon material were used to produce the modified CDs. Biomass raw materials, such as chitosan [11], waste wood chips [12] are more and more widely used due to its low cost, natural non-toxicity, and abundant sources. Cellulose, the most abundant

reserved biomass raw material on the earth, has been successfully applied in the fields of drug delivery, bio-imaging, catalysis and sensing [13, 14] due to its excellent degradability, renewable and bio-friendliness. As a carbon source, cellulose powder has also been used to synthesize CDs through high-pressure homogenization [15]. To improve the labeling brightness of CDs, cellulose-based biowaste of willow catkin was used to yield nitrogen and sulfur co-doped CDs (N/S-CDs) via the one-step combustion [16]. Usually, the mixture of cellulose and doping element contained chemicals in a state of solid powder were used as the raw materials to produce the modified CDs [17]. However, the uniformity and dispersity of the doping material need to be considered because it may have influence on the modification effect.

Compared to cellulose powder, the spatial network structure of cellulose hydrogel makes it be a better doping-material-carrier, and it has already been used in drug delivery. Thus, in present work, cellulose hydrogel was used as both the carbon source and the doping-materials-carrier to produce the N-doped CDs. The fluorescent properties of so-obtained CDs were characterized to confirm that whether the cellulose hydrogel system is competent to yield the element-doped CDs. In addition, the labeling properties of so-obtained CDs on living cells were quantitatively studied to find the correlation between the quantum yield (QY) of CDs and the imaging brightness. Finally, the cytotoxicity of so-obtained CDs was measured to see whether the N-doping will affect the biocompatibility of CDs.

2 Materials and methods

2.1 Materials and reagents

Cotton staple cellulose ($M_w = 8 \times 10^4$) was obtained from Hubei Chemical Fiber Group Co., Ltd., China. Epichlorohydrin (ECH), NaOH and urea (analytically grade) were purchased from Sinopharm Group, China. Human Amniotic Epithelial Cells and the cervical cancer cell line Hela cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences, China. Fetal bovine serum (FBS), MEM culture medium, PBS buffer 0.25% trypsin (containing EDTA) were supplied by Gino Biomedical Technology Co., Ltd, China. Cell Proliferation Cytotoxicity Detection Kit (CCK-8), ER-Tracker Red, Phalloidin TRITC and dialysis bags were purchased from Beyotime Biotechnology Co., Ltd., China. Millipore water was used for the entire experiment.

2.2 Synthesis of the cellulose hydrogel and CDs

The aqueous solution containing 7 wt% NaOH, 12 wt% urea and 81 wt% H_2O was precooled to $-20^\circ C$. Then cellulose was added to this solution, the mixture was stirred to dissolve the cellulose and finally form a 4% (wt%) transparent cellulose solution. Subsequently, a certain amount of ECH was dropped into the cellulose solution and incubated at $60^\circ C$ for 4 h to form the chemical cross-linked cellulose hydrogel [18]. After that, the cellulose hydrogel was rinsed in deionized water to thoroughly remove residual reagents to get the pure cellulose hydrogel.

To investigate the influence of alkali and urea on the QY and cell labeling property of CDs, the prepared cellulose hydrogel was treated in different ways before being used as the CDs source: (1) The prepared raw hydrogel was directly used to produce CDs without any treatment. In this case, "Cellulose CDs" was obtained. (2) The pure cellulose hydrogels were immersed in 7 wt% NaOH solution, 12 wt% urea aqueous solution, and 7 wt% NaOH plus 12% urea solution, respectively for 48 h and the CDs produced from these different ways were marked as "NaOH/cellulose CDs", "urea/cellulose CDs", and "NaOH/urea/cellulose CDs", respectively. The above 4 kinds of CDs were produced by one-step hydrothermal method, and the cellulose hydrogels in Teflon-lined stainless-steel autoclaves were incubated at $200^\circ C$ for 12 h. After centrifugation at high speed and ethanol extraction, the CDs with different particle sizes were collected via gradient dialysis (the cut-off molecular weights of dialysis bags were 14000D, 8000D, 3500D and 1000D). The CDs with different size were identified as the "> 14000D CDs", "8000D–14000D CDs", "3500D–8000D CDs", "1000D–3500D CDs" and "< 1000D CDs".

2.3 Characterization

The UV–visible absorption test was performed on a CARY-100 UV–Vis spectrophotometer (Agilent, USA) with a scan range of 200–800 nm for structural analysis of CDs.

To study the structure and functional groups of the CDs, (FT-IR) spectrometer (Nicolet Instruments, USA) was employed to obtain the FT-IR spectra by using a potassium bromide tableting method. X-ray photoelectron spectroscopy (XPS) spectra were recorded with ESCALAB 250XI (Thermo, USA).

The fluorescence spectra of the CDs were obtained by using a Hitachi F-2500 fluorescence spectrophotometer (Hitachi, Japan) with a scan range of 320–600 nm at a scan rate of 1500 nm/min.

JEM-2100F high-resolution transmission electron microscopy (HRTEM, Japan) was used to observe the morphology and microstructure of the fluorescent CDs.

2.4 Quantum yield

Relative fluorescence quantum yield of CDs was calculated according to the references [19, 20]: Quinine sulfate was dissolved in a solution of 0.1 M H₂SO₄ configured dilute solution ($n = 1.33$, the quantum yield = 0.54). By measuring the integrated fluorescence intensity of the CDs and the quinine sulfate solution at the same excitation wavelength and the absorbance of the excitation light for the wavelength (the absorbance are both less than 0.05), the fluorescence QY of the CDs can be calculated by the formula (1):

$$Y_u = Y_s \times \frac{F_u}{F_s} \times \frac{A_s}{A_u} \times \frac{\eta_u^2}{\eta_s^2} \quad (1)$$

where Y_u represents the QY value of CDs, F is the area of the fluorescence emission peak of, A is the absorbance at the excitation wavelength, η is the refractive index of the solvent (here, both η_s and η_u are 1.33). The subscript u denotes the sample, and s denotes the reference substance (here, quinine sulfate was used as the reference).

2.5 Cytotoxicity

The CCK-8 assay was used to evaluate the cytotoxicity of CDs. Human amniotic epithelial cells (AECs) were seeded in the 96-well culture plates with a density of 1×10^4 cells/cm² in MEM medium that containing 10% fetal bovine serum and 1% penicillin/streptomycin. After 6 h of incubation in a 37 °C, 5% CO₂ humidified incubator, all cells adhered on spread well on the bottom of the culture plate. Then the medium was removed and the cells were washed with phosphate buffer saline (PBS). 100 μ L new medium that containing different concentration of CDs (31.25 μ g/mL, 62.5 μ g/mL, 125 μ g/mL, 250 μ g/mL, 500 μ g/mL) were used to continue the cell culture. 12, 24 and 36 h later, the medium was removed again, and 100 μ L new medium plus 10 μ L CCK-8 solution was added into each well, and the cells were incubated in a 37 °C, 5% CO₂ humidified incubator under a dark condition for 2 h. Subsequently, the absorbance at 450 nm was measured by the microplate reader (Biotek Epoch2, USA). Cell viability (ρ) was determined by the following Eq. (2):

$$\rho = \frac{A}{A_0} \times 100\% \quad (2)$$

where A is the absorbance of sample wells, A_0 is the absorbance of control wells.

2.6 Cell imaging experiments

AECs and Hela cells were seeded in the confocal petri dishes with a density of 5×10^4 /cm² and incubated in the 37 °C, 5% CO₂ incubator for 24 h. Then, the old medium was replaced by the fresh medium that containing 500 μ g/mL CDs and further cultured for another 4 h. After being rinsed with PBS for 3 times (10 min for each time), the cells were immediately observed under the laser confocal microscope (LEICA TCS SP5, German) at the exciting wavelength of 405 nm. In the case that the cells co-stained with the endoplasmic reticulum, the CDs-incubated cells were first rinsed with HBSS twice and incubated in 1 μ mol/L ER-Tracker Red for 30 min at 37 °C. In the case that the cells co-stained with f-actin, CDs-cultured cells were fixed with 4% paraformaldehyde, then treated with 0.2% Triton-X, and finally incubated in 1 μ mol/L Phalloidin TRITC for 1 h at room temperature [21]. All fluorescent images of CDs labeling were recorded under the same camera parameters (scan area: 512×512 ; scanning speed: 400 Hz; pinhole size: 1 Airy) and the fluorescent brightness was quantitatively analyzed from these original images by means of Image J.

2.7 Data statistics analysis

All data are presented as the Mean $\pm$ SD, and the statistical analysis was performed by using SPSS 19.0. The statistical significances between data sets were expressed as p value or "Sig.". P or "Sig." < 0.05 was considered as statistically different, and P or "Sig." < 0.01 was considered as extremely significant in difference. The one-way ANOVA analysis was used for multiple comparisons and Sig. < 0.05 was considered as a significant difference. The Pearson correlation coefficient analysis was used to describe the correlation between the two variables. The closer the Pearson value to 1 is, the higher the correlation is.

3 Experimental results

3.1 Characterization of CDs

Figure 1a showed the UV-Vis absorption spectra of four kinds of CDs (NaOH/urea/cellulose CDs, NaOH/cellulose CDs, urea/cellulose CDs, and pure cellulose CDs). The distinct absorption peak at 272 nm was attributed to the π - π^* electronic transition of C=C. The weak wide shoulder around 315 nm was attributed to the n - π^* electronic transition of C=O [22]. The UV absorbance spectra indicated that the produced CDs contain chromogenic groups C=O, C=C.

The FT-IR spectra shown in Fig. 1b revealed more information about the groups in the CDs. Peaks in the range

Fig. 1 the UV-Vis absorption spectra **(a)**, and the FT-IR spectra **(b)** of the CDs

Table 1 Elemental composition of the CDs produced from different raw cellulose hydrogels

Sample name	C% (at%)	N% (at%)	O% (at%)
Cellulose CDs	73.39	2.18	24.42
NaOH/cellulose CDs	80.26	1.98	12.76
urea/cellulose CDs	73.32	7.51	19.17
NaOH/urea/cellulose CDs	68.68	6.54	24.78

of 3000–3500 cm^{-1} correspond to stretching vibration of O–H or N–H. The peak at 1431 cm^{-1} corresponds to the telescopic vibration peak of –OH. As a result of C–OH oxidation during the hydrothermal reaction, a C=O peak located at 1631 cm^{-1} was identified [23]. It was said that the C=O bond, as well as the graphite-like conjugated structure inside CDs, might be the luminescent donors in CDs. And a C=C stretching vibration peak located at 1594 cm^{-1} were detected. C=C is also regarded as a source of luminescence for fluorescence.

Compared to pure cellulose CDs, the C–O–C stretching vibration peak located at 1352 cm^{-1} appeared on the FT-IR spectra of NaOH/urea/cellulose CDs, NaOH/cellulose CDs and urea/cellulose CDs. The reason was as follow: when NaOH or urea was introduced into cellulose system, the ring-opening and oxidation effect of the cellulose glucose structural unit were promoted [4], and the C–OH were further oxidized by the residual oxygen atoms under the high temperature and pressure environment to yield more C–O–C in CDs [24]. In addition, an extra C–N characteristic absorption peak appeared at 1384 cm^{-1} in urea/cellulose

CDs and NaOH/urea/cellulose CDs, which indicated that N had been successfully doped into CDs in this case.

The XPS survey exhibited that the elements contained in the four kinds of CDs were mainly carbon, oxygen and nitrogen, and their relative contents were listed in Table 1. N had been successfully doped into CDs via introducing urea to the raw cellulose hydrogel, which showed a higher N content (7.75 at% in urea/cellulose CDs and 6.54 at% in NaOH/urea/cellulose CDs) than that of Cellulose CDs (2.18 at%) and NaOH/cellulose/cellulose CDs (1.98 at%). Approximately 2 at% of N in Cellulose CDs and NaOH/cellulose CDs may come from the residual urea in raw cellulose hydrogel. The C_{1s} spectrum of four kinds of CDs could be deconvoluted into 6 peaks (Fig. 2), which corresponded to C=C, C–C, C–N, C–O, C=O and O=C–O groups, respectively [20]. The proportion of each group listed in Table 2 indicates that the ratio of C–N in the urea/cellulose CDs and the NaOH/urea/cellulose CDs are much higher than the other two kinds of CDs, and the ratio of C=C or C=O in the NaOH/urea/cellulose CDs are also relatively high. Since C–N, C=C and C=O are regarded as the luminescence sources for fluorescence [25], NaOH/urea/cellulose CDs was expected to exhibit a better luminescent effect.

Figure 3 is the color contour map of the fluorescence intensity distribution of NaOH/urea/cellulose CDs (50 $\mu\text{g}/\text{mL}$), and that of the other three kinds of CDs exhibited a similar appearance. The longitudinal coordinate of the map represents the excitation wavelength, and the horizontal ordinate represents the emission wavelength. From this map, the distinct optical property of CDs redshift effect [26] was observed. When the excitation wavelength increased from 300 to 400 nm, the emission wavelength

Fig. 2 The C1S XPS spectra of the CDs **Table 2** Proportion of groups of the CDs that produced from different raw cellulose hydrogels

Sample name	C=C%	C-C/C-H%	C-N%	C-OH/C-O-C%	C=O%	O=C-O%
Cellulose CDs	14.93	36.76	4.99	16.18	12.86	14.29
NaOH/cellulose CDs	21.92	26.46	3.76	30.64	8.62	8.59
urea/cellulose CDs	20.05	28.54	18.30	19.13	8.96	5.02
NaOH/urea/cellulose CDs	18.88	21.61	16.62	17.02	14.17	11.69

Fig. 3 Color contour map of fluorescence intensity distribution of NaOH/urea/cellulose CDs (50 µg/mL)

Table 3 The optimal excitation and emission wavelengths of CDs produced from different raw cellulose hydrogels

Sample name	Particle size (identified by the molecular weight of the dialysis bag)	Excitation wavelength (nm)	Emission wavelength (nm)
Cellulose CDs	< 14000D	370	464
NaOH/cellulose CDs	< 14000D	370	463
urea/cellulose CDs	< 14000D	370	470
NaOH/urea/cellulose CDs	< 14000D	370	469
	> 14000D	370	473
	8000D–14000D	370	470
	3500D–8000D	370	469
	1000D–3500D	370	461
	< 1000D	370	457

(a) **(b)****Fig. 4** HRTEM images of NaOH/urea/cellulose CDs (the four images separately corresponded to the morphology of the “< 1000D CDs”, the morphology of the “8000D–14000D CDs”, the microstructure of

the CDs, and the fast Fourier transform (FFT) pattern) **(a)**; and the distribution of particle size after dialysis **(b)**

red-shifted accordingly from 400 to 600 nm, which implied that the emission wavelength of CDs is excitation wavelength dependent. The fluorescence of CDs can be excited by a wide range of wavelengths (300–400 nm), which means that it might be relatively easier to be traced. The optimal excitation and emission wavelengths of CDs can also be determined from this map, and the results were listed in Table 3. Although the emission wavelength of the four kinds of CDs was slightly different, the optimal excitation wavelengths were the same (370 nm), which agreed with some previous reports [27]. The particle size of CDs affected its optimal emission wavelengths (Table 3).

As the particle size increased, the emission wavelength red-shifted. This is because the larger the particle size of CDs is, the more conjugated structures contained in CDs, which resulted in a redshift of fluorescence emission wavelength [28].

Figure 4a showed the morphology and microstructure of NaOH/urea/cellulose CDs that observed by means of HRTEM. From left to right, the four images separately corresponded to the morphology of the “< 1000D CDs”, the morphology of the “8000D–14000D CDs”, the microstructure of the CDs, and the fast Fourier transform (FFT) pattern. The CDs were nearly spherical in shape, and the

Fig. 5 Influence of the raw cellulose hydrogels (mean $\pm$ SD, $n=3$, $*P<0.05$ and $**P<0.01$, compared to cellulose CDs) (a) and the particle size (b) on the fluorescence quantum yield (QY) of CDs

particle size of “the 8000D–14000D CDs” was less than 10 nm, and the particle size of the “< 1000D CDs” was even smaller. The as-prepared CDs have a high crystalline structure with well-resolved lattice fringes with the relative interplanar distance around 0.22 nm, corresponding to that of the (001) plane of graphite [29]. The corresponding FFT pattern also demonstrated that the CDs were polycrystalline structured. There was no morphology and structure difference between the 4 kinds of CDs that prepared in this work.

The particle sizes of CDs collected through dialysis bags with different cut-off molecular weights were counted, and the size distribution was plotted and Gaussian fitted (Fig. 4b). As the molecular weight of the dialysis bag decreases, the diameter distribution curves of CDs shift leftward. Corresponding to the “8000D–14000D CDs”, the “3500D–8000D CDs”, the “1000D–3500D CDs” and the “< 1000D CDs”, the mean diameter of CDs are 4.79 ± 1.04 nm, 3.94 ± 1.21 nm, 3.24 ± 0.64 nm and 2.41 ± 0.93 nm, respectively.

3.2 Quantum yield of CDs

Relative fluorescence quantum yield (QY) is an index to characterize the fluorescence properties of a fluorescent substance. Generally, the value of QY is less than 1, and the higher the value is, the stronger the fluorescence would be excited to form the substance. Figure 5 showed the relative fluorescence QY value of the prepared CDs in this experiment. When the particle size remained no difference among the 4 kinds of CDs, the relative fluorescence QY values decreased in the following sequence

(see Fig. 5a): NaOH/urea/cellulose CDs > urea/cellulose CDs > NaOH/cellulose CDs > pure cellulose CDs. One-way ANOVA analysis result showed that a significant difference existed among the 4 kinds of CDs (Sig.=0.000). The QY of the CDs that made from the pure cellulose hydrogel is the lowest (0.0542 ± 0.0030). When NaOH or urea was introduced into the raw cellulose hydrogel, the QY of CDs was significantly improved (Sig.=0.000, compared to Cellulose CDs). This is because the existence of NaOH allowed the cellulose chains being more easily broken [4]. As a result, finer structures such as C=C and C=O were formed during the hydrothermal process to provide more sources of fluorescent emission. The existence of urea in the raw cellulose hydrogel let nitrogen atoms successfully grafted to the CDs, and the nitrogen-containing group is regarded as an auxochrome group who can improve the fluorescence emission efficiency by providing electrons [25]. Both NaOH and urea in raw cellulose hydrogel were beneficial to the QY of CDs, NaOH/urea/cellulose CDs showed the highest QY among the four CDs, which was 0.1965 ± 0.0013 . One-way ANOVA analysis result exhibited an extremely significant difference among the four kinds of CDs (Sig.=0.000). That means, the carried NaOH and Urea in raw cellulose hydrogels could effectively improve the photoluminescence of so-obtained CDs.

Figure 5b revealed the influence of particle size on the QY of CDs that produced from the same raw cellulose hydrogel. The one-way ANOVA analysis result indicated that the particle size will affect the QY of CDs (Sig.=0.000). The smaller the average particle size is, the higher the QY value is. This is because the smaller quantum dots have a larger proportion of the surface luminescent groups [30].

Fig. 6 The fluorescent images of HeLa cells that labeled by NaOH/urea/cellulose CDs with different size **(a)**; and labeled by CDs (“the < 1000D CDs”) that produced from different raw cellulose hydrogels (mean $\pm$ SD, $n = 3$, $^*P < 0.05$ and $^{**}P < 0.01$, compared to the cellulose CDs) **(b)**

3.3 Living cell labeling

Figure 6a showed the fluorescent images of HeLa cells labeled by NaOH/urea/cellulose CDs with different particle size. Compared to the CDs with particle size > 14000D, the labeling brightness of the CDs with the particle size < 1000D is almost 5.5 times higher (the average gray value of labeled cells was 6.10 ± 1.18 vs. 34.12 ± 6.66 , and the Sig. of t test given by SPSS was 0.000). This implies that the labeling brightness of CDs is size-dependent. Therefore, following cell labeling experiments were performed under the condition that the particle size of CDs was < 1000D.

For the “< 1000D CDs”, the labeling brightness showed significant difference among the 4 kinds of CDs (Fig. 6b, the Sig. given by one-way ANOVA is 0.000). The labeling brightness of pure cellulose CDs was the lowest (the average gray value was 13.49 ± 2.79). When NaOH was carried in cellulose hydrogel as the raw material, the labeling

brightness of so-obtained CDs trends to be improved, but there was no statistical difference (Sig. = 0.216, compared to Cellulose CDs). When urea was applied to the raw cellulose hydrogel, the labeling brightness of so-obtained CDs on HeLa cells was significantly increased (Sig. = 0.000, compared to Cellulose CDs), and the NaOH/urea/cellulose CDs had the highest labeling brightness among the 4 kinds of CDs (Sig. = 0.000, compared to Cellulose CDs).

In present study, the QY of CDs increased when NaOH and urea were added into the cellulose hydrogels, and the corresponding photoluminescence intensity increased to yield brighter images. The smaller the particle size of CDs is, the higher the QY is, and the brighter the labeled image is. In addition, the cellular imaging effect of CDs is not only determined by quantum efficiency, but also by the uptake rate of cells. Studies have found that the uptake of nanoparticles by cells is determined by several factors such as the size, shape, specific surface area, surface charge and

Fig. 7 The images of AECs and Hela cells labeled by NaOH/urea/cellulose CDs (the “< 1000D CDs”)

Fig. 8 Intracellular distribution of CDs. Hela cells were first incubated with the NaOH/urea/cellulose CDs (the “< 1000D CDs”) for 1 h and then applied with endoplasmic reticulum marker for 30 min (a); with actin marker for 2 h (b)

surface modification of the nanoparticles. It is said that nanoparticles with smaller size are more easily to be taken up by cells, and the doping of nitrogen causes more amino groups on the surface of quantum dots, making quantum dots more readily taken up by cells [31, 32]. Therefore,

NaOH/urea/cellulose CDs with the smallest particle size (the “< 1000D CDs”) contributed to the brightest cell labeling image in this work.

Figure 7 shows the laser confocal microscope images of living AECs and Hela cells that co-incubated with NaOH/

urea/cellulose CDs (the “< 1000D CDs”) for 4 h. The bright-field images indicated that both cells grew well, the adhesion shape of the cells kept normal, which implies that CDs has not significant cytotoxicity and is suitable for living cell’s marking. The dark field images reveal the fluorescent labeling result of CDs. Under the same labeling condition, the fluorescent image of Hela cells is brighter than that NaOH/urea/cellulose CDs of AECs (the average gray value of labeled cells was 11.45 ± 2.25 vs. 34.12 ± 6.66). This might because Hela cells are cancer cells while AECs are normal cells. The endocytosis of cancer cells is stronger than that of normal cells, which result in a more uptake of CDs and consequent stronger labeling brightness. From the merged images, it also can be seen that CDs fluorescent almost covered the whole area of Hela cells.

Further observation on CDs distribution in Hela cells was performed by co-staining with endoplasmic reticulum and f-actin (see Fig. 8). F-actin forms the cytoskeleton and the pseudopodia and microvilli on the cell membrane

surface, and the endoplasmic reticulum is a cytoplasmic-membrane system that externally connected to the cell membrane and the outer layer of the nuclear membrane. The CDs evenly dispersed in the area bounded by f-actin, and rarely distributed in the membrane system, including cell membrane, cytoplasmic membrane and nuclear member. No CDs was found in pseudopodia, either. These indicate that CDs immobilized in the cytoplasm, and contributed to a non-specific fluorescent labeling. Specific binding molecular needs to be grafted on CDs if the specific labeling is expected.

The labeling brightness of NaOH/urea/cellulose CDs (the “< 1000D CDs”) is similar to that of ER-tracker (the average gray value of labeled cells was 34.62 ± 8.96 and 34.12 ± 6.66 , respectively) on living cells. Compared to TRITC, the labeling brightness of trends to be weaker. The part reason may come from the CDs loss during the Triton treatment and subsequent rinse period of TRITC staining. In addition, the optimal exciting wavelength of CDs

Fig. 9 The cell proliferation curve of control groups (a) and the cell viability after the “< 14000D CDs” treated for 12 h (b), 24 h (c) and 36 h (d) (n = 3; *: $P < 0.05$, compared to the control groups)

produced in this work is 370 nm, however, the exciting wavelength of laser confocal microscope is 405 nm, which leads to a weaker emission than it could be.

3.4 Cytotoxicity of CDs

The cell viabilities after being treated by CDs (the “< 14000D CDs”) were shown in Fig. 9. Figure 9a was the proliferation curve of AECs without any treatment. It indicated that the AECs grew well during 36 h of incubation. Figure 9b and c showed the viability of AECs that treated with different concentration of CDs for 12 h, 24 h and 36 h, respectively. When the exert concentration of CDs was lower than 250 $\mu\text{g/mL}$, no significant cytotoxicity was found within 36 h. When the exert concentration of CDs was as high as 500 $\mu\text{g/mL}$, except the pure cellulose CDs, the other three kinds of CDs showed a significant decrease in cell viability for 36 h. The viability of urea/cellulose CDs and NaOH/urea/cellulose CDs is slightly lower than that of pure cellulose CDs might because the doped N element has concentration-dependent cytotoxicity on cells [33], which is consistent with the results of other carbon nano-materials such as graphene oxide [34, 35]. However, when the exert concentration of CDs was as high as 500 $\mu\text{g/mL}$, the cell viability remained over 80% treated with CDs for 36 h. The cytotoxicity experiment results implied that the CDs prepared from hydrogels can be potentially used for living cells’ labeling, especially when the exert concentration is lower than 500 $\mu\text{g/mL}$.

4 Discussion

In present work, N-doped CDs have been successfully obtained by introducing urea to cellulose hydrogel as the raw material. As a carbon source, cellulose hydrogel is more beneficial to producing CDs when compared to that of the cellulose powder. During the preparation of cellulose hydrogel, NaOH in the alkali-urea system effectively destroyed the hydrogen bond between cellulose molecules. The urea in alkali-urea system encapsulated carbon chain to prevent it from self-aggregating [36, 37]. As the inclusion mode showed in Fig. 10a, the cellulose chain is buried in the inclusion, and the NaOH and urea hydrates attached to the periphery of the cellulose chain.

Fig. 11 The correlation between the QY of the CDs and the labeling brightness of Hela cells that obtain from Pearson analysis in SPSS

Fig. 10 Schematic depiction of the dissolution of cellulose in aqueous NaOH/urea solutions (a); and the spatial network structure of NaOH/urea cellulose hydrogel (b)

Thus, cellulose can be well dissolved and need crosslinking agent to help it to form the network-structured hydrogel. The broken of hydrogen bond between cellulose molecules will be beneficial to subsequent CDs' synthesis though the hydrothermal incubation. Therefore, the CDs prepared in this work was regular in size and shape. As a doping-material-carrier, the spatial network structure of cellulose hydrogel let it have many distinct advantages on producing element-doped CDs. First, the spatial network of hydrogel provides space for doping materials (should be aqueous), allow them evenly to disperse in the network, and even attach to the cellulose chain (Fig. 10b). Thus, the doping efficiency could possibly be improved. Second, the doping dose can be easily controlled by adjusting the concentration of the doping-material in the aqueous solution. Third, the doping material was not limited to urea and NaOH, theoretically, all kinds of aqueous soluble materials can be introduced to the hydrogel network to provide grafting groups to CDs. Cellulose hydrogel is a novel material to produce CDs and element-doped CDs.

In present work, the so-obtained CDs exhibited a non-specific labeling on living cells, and the labeling brightness is determined by both the QY value of the CDs and the endocytosis of the living cells. For the same cell line, the labeling brightness is mainly QY dependent. Figure 11 showed the correlation between the QY of the CDs and the labeling brightness of Hela cells (the data is corresponding to Fig. 6) by means of Pearson analysis in SPSS. The analysis result indicated that the two variables (QY and labeling brightness) are significantly correlated (Pearson value = 0.907, Sig. = 0.033), and the relation between QY and Labeling brightness can be linearly fitted with $R^2 = 0.823$. This means that the labeling brightness of the same cell line is nearly proportional to the QY value of the prepared CDs. Therefore, QY of the CDs could be used as the index to describe its labeling brightness, and it could possibly be used in preliminary screening of photoluminescence materials without cell imaging experiment.

5 Summary

In present work, cellulose hydrogel was used as the carbon source and the doping-material-carrier to produce CDs and N-doped CDs. The groups in so-obtained CDs were measured by means of UV-Vis absorption spectrum, FT-IR spectrum and XPS to confirm whether the N element has been successfully doped in CDs. The microstructure, the morphology, and the particle size of the CDs were observed by means of HRTEM. The QY value of the CDs was detected to describe its photoluminescent property. The labeling brightness was used as the index to investigate the influence of N-doping, particle size, as well as the

cell line on the bioimaging property of the CDs. Finally, the influence of element-doping on the cytotoxicity were measured. The experimental results showed that:

Cellulose hydrogel is a nice carbon source and doping-material-carrier to produce fine N-doped CDs due to its distinct spatial network structure. The urea carried in cellulose hydrogel network achieved to dope N into the CDs, and subsequently improved the QY of the CDs through providing the nitrogen-contained auxochrome group. The fluorescence QY values of 4 kinds of CDs produced in this work decreases in the following sequence: NaOH/urea/cellulose CDs > urea/cellulose CDs > NaOH/cellulose CDs > pure cellulose CDs. For the same kind of CDs, the smaller the particle size is, the higher the QY value is.

The CDs immobilized in the cytoplasm of the living cells, and contributed to a non-specific fluorescent labeling. The labeling brightness is both the QY value of the CDs and the uptake rate of the living cells dependent. For the same cell line, the QY of CDs and the labeling brightness of living cells are significantly linear correlated.

The cytotoxicity CDs is low enough for a long-time observation on living cells.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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