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Research progress in the use of cationic carbon dots for the integration of cancer diagnosis with gene treatment

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Abstract: As a type of carbon dot (CD) with a positive charge on their surface, cationic carbon dots (CCDs) can be obtained from CDs and amino-containing cationic compounds by one-step or two-step preparation. They not only retain the good fluorescence performance, low toxicity and biocompatibility of CDs, but also improve their gene delivery efficiency and cell uptake capacity. These excellent properties give CCDs potential advantages in the fields of the targeted fluorescence imaging of cancers and gene therapy. This paper reviews the preparation methods and properties of CCDs, suggesting that they can be used as good targeting carriers for imaging cancer and gene therapy. In addition, the basic principles of CCDs for cancer detection and treatment, and their uses in integrated cancer diagnosis and gene therapy are introduced. Current problems and future development trends of CCDs for this purpose are discussed.

Key words: Cationic carbon dots; Gene therapy; Fluorescent imaging; Integration of cancer diagnosis and treatment

1 Introduction

Global cancer data show that cancer deaths in China accounted for 30% of the global deaths in 2018, ranking the first in the world[1]. One of the major reasons is that most cancers have no obvious symptoms in the early stage and the detection rate of small lesions is low with traditional examination methods, which are failed to seek medical treatment in time. When patients have obvious symptoms or lesions are detected, cancers are often developed in the middle and late stages, seriously threatening people's life[2–3]. Therefore, it is imperative to explore an early diagnosis and treatment of cancer in clinical work.

At present, according to the characteristics and location of cancer, the diagnosis of cancer mainly relies on imaging examination and endoscopy, such as X-ray, computed tomography (CT), magnetic resonance imaging (MRI), positron emission computed tomography (PET-CT), gastrointestinal endoscopy. It is difficult to find early lesions because some examinations are not sensitive to the diagnosis of tumors[4]. The treatment methods for cancers mainly include surgical resection, radiotherapy, chemotherapy and other methods. However, they all have some limitations. Surgical resection is very traumatic, and the tumor mass is not thoroughly removed, which can cause recurrence or metastasis[5]. Radiotherapy has poor curative effect on malignant tumors that are not sensitive to radiation and can damage normal tissues around cancer tissues, causing multiple organ exhaustion[6]. Chemotherapy has serious toxic and side effects on human body because of the lack of specificity. Meanwhile, chemotherapy drugs will gradually develop resistance and reduce the therapeutic effect[7].

As a new cancer treatment method, gene therapy is favored by researchers. It refers to the treatment of cancer by genetic transfer techniques that allow the gene transfer technology to inset the appropriate receptor cells of cancer patients to correct or compensate for genetic defects. Gene therapy methods can well avoid the disadvantages of traditional treatment

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methods and can precisely express and inhibit cancer cell proliferation. However, the exogenous genes are difficult to reach the cancerous tissue because they are easily eliminated by blood. In addition, the negatively charged nature of genes makes them more difficult to enter cancer cells through cell membrane[8–9].

Based on the above problems, researchers have developed a class of nano-transport materials, such as carbon dots (CDs), chitosan (CS), liposomes, metal nanoparticles. Most of them have excellent biocompatibility, good gene loading rate, easy surface modification and other good properties for gene delivery[10–13]. CDs, which are a class of carbon-based zero-dimensional materials with the particle sizes of less than 10 nm, have many advantages, such as low toxicity, high quantum yield, low cost, environmentally friendly, wide source of raw materials, good water solubility, excellent fluorescence luminescence performance and so on[14–20]. The excellent fluorescence luminescence performance of CDs makes them stand out from many delivery materials. which can effectively realize the fluorescence imaging of cancer cells for early cancer diagnosis. But the gene loading capacity of CDs is not satisfactory[21].

Cationic carbon dots (CCDs) contain a large number of positively charged groups on CD surface, which can well solve the problem of negatively charged gene loading, while also retaining the excellent fluorescence and biocompatibility properties of CDs. At present, CCDs are mainly prepared by a one-step method with cationic compounds containing amino ($-NH_2$) or by a two-step method with CDs and cationic compounds containing $-NH_2$, thus obtaining this type of CCDs containing $-NH_2$ on the surface. In addition, the specific targeting molecules grafted on the surface of CCDs can recognize and specifically bind to the receptors on the surface of cancer cells, thereby realizing the integration of cancer-targeted fluorescent imaging diagnosis and gene therapy (Fig. 1).

This review summarizes the preparation methods of CCDs, and briefly introduces their properties and summarizes their research progress on applications in cancer diagnosis and gene therapy.

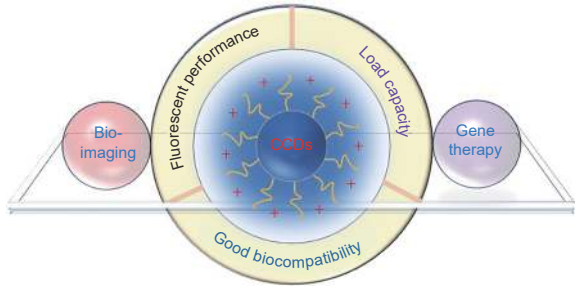

Fig. 1 The properties of CCDs and their application in cancer fluorescence imaging diagnosis and gene therapy.

2 Preparation of CCDs

In general, according to the carbon source, the preparation of CCDs can be divided into “top-down” and “bottom-up” methods. The “top-down” method refers to the physical or chemical shearing and oxidation of large carbon sources (carbon nanotubes, carbon fibers, graphite rods, graphene, and activated carbons) to produce CCDs with small particle sizes, such as arc discharge, laser ablation, and electrochemical oxidation[22–26]. The “bottom-up” method uses citric acid (CA), fructose, protein, urea, vitamins and other small molecular carbon sources to prepare CCDs by the hydrothermal method, microwave method, pyrolysis method, template method and so on[27–30].

CCDs have a large number of positively charged amino groups ($-NH_2$) on their surface. According to the different methods to obtain $-NH_2$, the preparation of CCDs can be divided into two types (Fig. 2). A one-step method, where cationic compounds are directly used as the carbon source to inherit the cationic compound $-NH_2$. A two-step method, where CDs are firstly synthesized, and then CCDs are prepared by passivating the surface of cationic compounds and preserving $-NH_2$.

2.1 One-step preparation

The one-step method is the simultaneous formation of CDs and surface passivation by $-NH_2$ to obtain CCDs. The preparation of CDs is usually completed through a series of reactions, such as ionization condensation, low-temperature polymerization and high-temperature carbonization. The active structure of reactants is likely to be embedded on the surface of CDs, so that as-prepared CDs inherits some specific

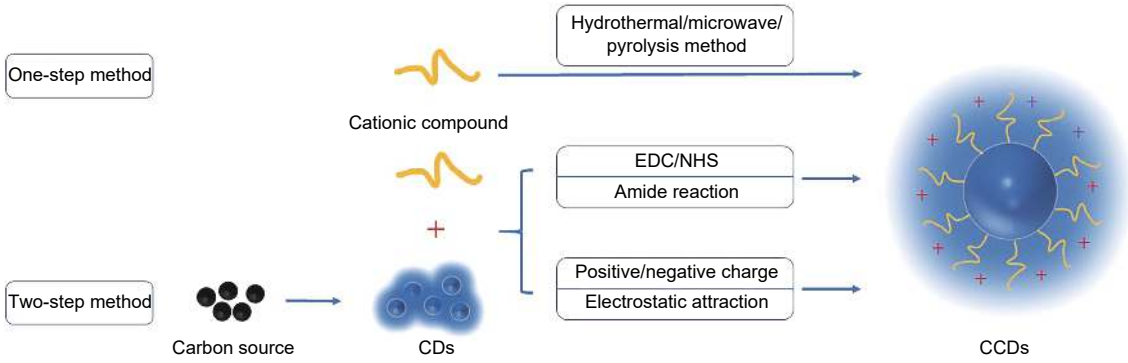

Fig. 2　Schematic diagram of preparation CCDs by one-step and two-step methods.

properties of reactants[31]. Therefore, for the one-step method to prepare CCDs, the carbon source should have active structural substances rich in positive charges. Such substances are cationic compounds, mainly including polyethyleneimine (PEI), polyethylene glycol (PEG), polylysine (PLL) and polyethylene diamine (PAMAM) and other amine-containing substances. The following is mainly to introduce the microwave, pyrolysis and hydrothermal one-step synthesis of CCDs, and summarizes the various characterization data of CCDs synthesized in the references (Table 1).

Table 1　Summary of CCDs prepared by the one-step methods.

Method	Carbon source	Zeta (mV)	QY (%)	Size (nm)	Fluorescent color	Ref.
Microwave	Glucose, Arginine	+21.8±0.2	12.7	1−7	Blue	[32]
	glycerol, PEI	+24	9.4	<50	Blue	[33]
Pyrolysis	Spermidine	+45	3.85	6.33±1.35	Blue	[34]
Hydrothermal	Malic acid, PEI	+26	41	1.2−5.1	Green	[35]
	Cetrimonium bromide	+10	20	1−2.5	Blue	[36]

2.1.1　The microwave method

The microwave method is to obtain CCDs by carbonizing a cationic compound or the mixture of a carbon source and a cationic compound under microwave radiation assisted heating. Cao et al[32] used arginine and glucose as reactants that were heated in a household microwave oven at 700 W for 10 min to obtain blue fluorescent CCDs. The fluorescence quantum yield (QY) was 12.7%, and the particle size distribution was 1−7 nm. Fourier transform infrared spectroscopy (FT-IR) analysis showed that the vibration broad absorption bands of carbon-nitrogen bond (N−H) and hydrogen-oxygen bond (O−H) groups appears from 3 000 to 3 410 cm^{-1}, and there was a strong peak caused by carbon-oxygen double bond (C=O) at 1 630 cm^{-1}, which proved carbonization reactions. The $-NH_2$ in arginine remained on the surface of CDs, thus CCDs were successfully prepared. Liu et al[33] synthesized yellow-brown CCDs using a mixture of glycerin and branched polyethyleneimine (bPEI) as raw materials in a 700 W household microwave oven, which emitted blue fluorescence under ultraviolet light at 365 nm with a fluorescence quantum yield of 9.4% and particle sizes of 4−12 nm. It was found from FI-IR that amide groups (−CO−NH−) were introduced into CCDs, suggesting the formation of CCDs.

2.1.2　The pyrolysis method

CCDs were obtained by the pyrolysis method through carbonizing cationic compounds as carbon sources at a high temperature. Jian et al[34] used spermidine (SPD) powder as a raw material, which was directly pyrolyzed to produce CCDs by simple dry heat treatment. The product was measured by Zeta potential and showed about +45 mV. X-ray photoelectron spectroscopy (XPS) found that N 1s XPS spectrum showed two types of nitrogen bonds, C=C−N (400.8 eV; 65.8%) and C−NH−C (399.4 eV; 34.2%). The FT-IR spectrum clearly showed that it had a structure similar to the original spermidine. These characterization methods of chemical substances can verify the successful synthesis of CCDs with a size of 6.33±1.35 nm. It emitted blue light under ultraviolet light, and the fluorescence quantum yield was 3.85%.

2.1.3　The hydrothermal method

CCDs were prepared by the hydrothermal meth-

od by mixing cationic compounds and water in a closed autoclave followed by reactions at high pressure and high temperature. Guo et al[35] prepared CCDs using 0.5 g of malic acid and 0.5 g of PEI as raw materials at 180 ℃ for 10 min. The measurement of Zeta potential showed that CCDs had a positive charge, which was consistent with the polycationic properties of PEI. In addition, FT-IR analysis showed that a strong peak at 1 707 cm^{-1} derived from −CO−NH− appeared, indicating that −COOH of malic acid reacted with $-NH_2$ of PEI to form −CO−NH−. At the same time, the $-NH_2$ vibration peak of PEI was found, demonstrating that PEI was grafted onto the surface of CCDs, which proved the successful preparation of CCDs. The particle size distribution was 1.2−5.1 nm, the fluorescence quantum yield reached 41%, and the product emitted green fluorescence. Saberi et al[36] used cetyl ammonium bromide (CTAB) as a raw material at 180 ℃ for 6 h to easily synthesize blue fluorescent CCDs. Similarly, FT-IR and Zeta potential were used to indicate the presence of cationic $-NH_2$, confirming the synthesis of CCDs, with a particle size of 1−2.5 nm, a fluorescence quantum yield of about 20%, and blue light emission.

The one-step method for preparing CCDs combines the synthesis of CDs and the modification of passivators together by a simple and rapid way. In the whole preparation process, no matter what preparation method is used, the retention of $-NH_2$ structure in the carbon source is the key to the successful preparation of CCDs. At present, the CCDs prepared by the hydrothermal method have the advantages of uniform particle size distribution and simple operation, which is more favored by researchers.

2.2 Two-step preparation

For CCDs prepared by a two-step method, CDs containing −COOH and hydroxyl (−OH) on their surface, are first prepared by the hydrothermal method, pyrolysis method or microwave assisted method. Then the positively charged CCDs are obtained by the cationic compounds with a high density of positive charge $-NH_2$ grafted on the surface of as-prepared CDs by electrostatic attraction or amide reaction. In the following, the two-step method for preparing CCDs is introduced by amide reaction and electrostatic attraction. The properties of CCDs from two-step preparation are shown in Table 2.

Table 2 Summary of CCDs prepared by the two-step methods.

Method	Carbon source	Zeta (mV)	QY (%)	Size (nm)	Fluorescent color	Ref.
Amide reaction	CA, Urea, bPEI	+24	18.7	10	Blue	[37]
	CA, EDA, bPEI	+46.68± 0.78	-	22.8±6.7	Blue	[38]
Electrostatic attraction	Candle soot, PEI	+40	-	<8.1	-	[39]
	CA, EDA,	+15.6	-	122.7	Blue	[40]

2.2.1 Amide reaction

In amide reaction of CDs, the stable −CO−NH− for CCDs is obtained by the reaction of −COOH on the surface of CDs and $-NH_2$ in the cationic compound under the action of a catalyst. Cui et al[37] used a 1∶2 ratio of CA to urea as carbon sources to prepare CDs rich in −COOH on their surface by a microwave method. Then, under the catalysis of carbodiimide/N-hydroxysuccinimide (EDC/NHS), CCDs were obtained by CDs reacting with bPEI. Moreover, the tumor suppressor gene p53 and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can be combined by electrostatic attraction on CCDs in vitro, which effectively protects p53 and TRAIL from being degraded by serum to realize the efficient delivery of p53 and TRAIL. Similarly, Shu et al[38] synthesized CDs by a one-step hydrothermal method using CA and ethylenediamine (EDA) as carbon sources, and then CCDs were obtained by grafting bPEI onto the surface of CDs through amide reaction (Fig. 3a). FT-IR spectroscopy confirmed that bPEI was successfully modified on the surface of CDs, and positively charged CCDs can effectively bind to genes, demonstrating their high-efficiency targeting delivery for small interfering RNA (siRNA)-mediated ability to cancer cells.

2.2.2 Electrostatic attraction

CDs riched in −COOH and −OH can be combined with cationic compounds through electrostatic interaction. Liu et al[39] mixed the synthesized CDs and PEI into a flask, heated to 90 °C, stirred vigor-

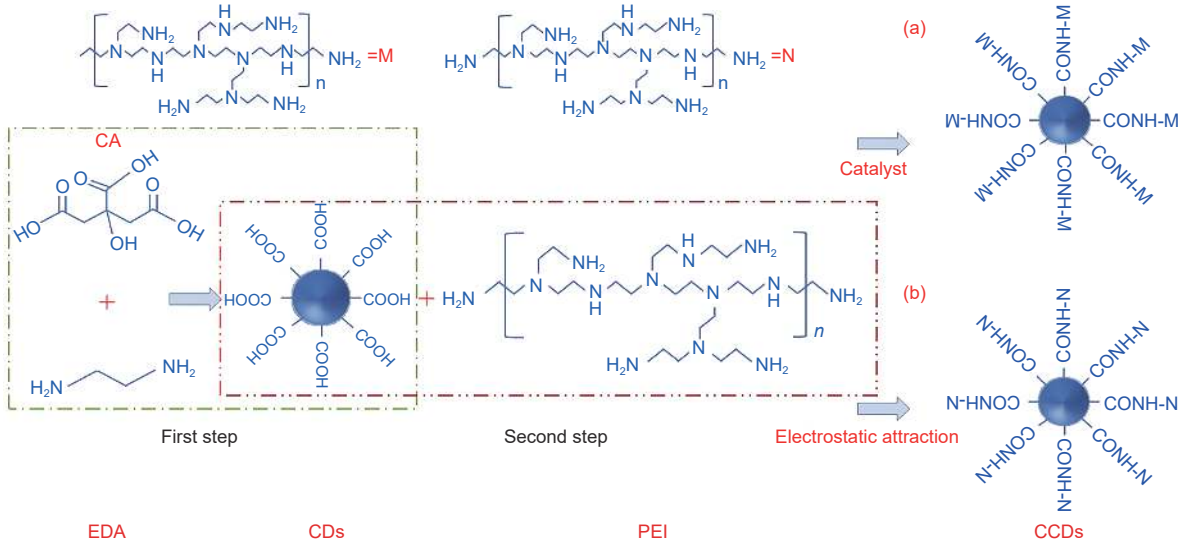

Fig. 3　The principle of preparing CCDs by the two-step method.

ously, and then cooled and dialyzed to obtain CCDs. The FT-IR spectra analysis showed that there were no new absorption bands other than the characteristic absorption bands of CDs and PEI after passivation modification. Therefore, it is speculated that CCDs can be prepared by encapsulating CDs with PEI through electrostatic adsorption. Kong et al[40] synthesized CDs from CA and EDA by a hydrothermal method, and then assembled CDs into CCDs by stirring with PEI at room temperature. The sizes of CCDs are much bigger than CDs, as observed under a high resolution transmission electron microscope, which is caused by PEI package. Moreover, the Zeta potential of CCDs is +15.6 mV while that of CDs is −20.3 mV, demonstrating the feasibility to prepare CCDs by electrostatic adsorption from CDs (Fig. 3b).

Both amide reaction and electrostatic attraction can be used to coat cationic compounds on the surface of CDs to form CCDs. For CCDs obtained by amide reaction, the formation of −CO−NH− bond requires the action of a catalyst under controllable reaction conditions, suggesting that it is a little complicated. In contrast, the electrostatic attraction assembly of CCDs has the advantages of simplicity, but the electrostatic attraction force is relatively weak and unstable.

In general, both the one-step method and the two-step method can preserve the $-NH_2$ of cationic compounds. The prepared CCDs not only change the surface state of CDs and enhance their fluorescence, but also play a positive role in gene carrying, making CCDs have a more important position in the field of fluorescence imaging of cancer cells and cancer gene therapy. Among them, in the two-step method of preparing CCDs, the first step is to synthesize CDs rich in −COOH and −OH on their surfaces, which is beneficial to the electrostatic action or passivation of the cationic compound in the second step. The direct modification by a passivating agent on the surface of CDs can better retain $-NH_2$ to CCDs, avoiding the loss of $-NH_2$ during the one-step preparation process. However, the two-step method is complicated, while the one-step method is relatively simple and rapid.

3　Properties of CCDs

To achieve the dual-mode integration of the cancer diagnosis and gene therapy, for the probes and carriers, their good biocompatibility and low toxicity are the foundation, their high sensitivity and high resolution inspection methods are the key, and their strong gene loading capacity is also particularly important. Because CCDs have good biocompatibility and excellent fluorescence performance, they can be used for fluorescence imaging. Fluorescence imaging is a technique in which a confocal laser scanning microscope receives the fluorescence signal emitted by the fluor-

escent substance after being excited, showing high sensitivity and high resolution[41]. In addition, the surfaces of CCDs contain a large amount of positively charged $-NH_2$, which gives them a strong gene loading capacity. These characteristics are enough to make CCDs a good carrier for the integration of cancer diagnosis and gene therapy.

3.1 Good biocompatibility and low toxicity

Good biocompatibility and low toxicity are the basis of CCDs for cancer diagnosis and gene therapy. Abdallah et al[42] found that CCDs had a large surface cation density, and excessive $-NH_2$ can damage cell membranes. Hasanzadeh et al[43] also confirmed that the more the free $-NH_2$, the lower the cell survival rate, the cell survival rate (from 98% to 90%) decreased with increasing the concentration of CCDs (from 15.625 to 500 mg/mL). Therefore, it is important to have an appropriate amount of $-NH_2$ on the surface of CCDs.

The appropriate $-NH_2$ on the surface of CCDs can achieve a low toxicity by controlling the reaction conditions during the synthesis of CCDs. Liu et al[33] controlled the cytotoxicity of CCDs by adjusting the pyrolysis time. In the appropriate pyrolysis time, the obtained CCDs had a lower cytotoxicity, which may be related to the destruction of $-NH_2$ on the surface of CCDs during the pyrolysis of PEI. Therefore, the prepared CCDs can be used for biological experiments.

3.2 Fluorescence performance

3.2.1 Photoluminescence and fluorescence stability

Photoluminescence and fluorescence stability are the most basic optical properties of CCDs used in biological fluorescence imaging. CCDs not only can inherit the photoluminescence characteristics of CDs, but also show a higher photoluminescence activity, which is attributed to a higher efficiency of exciton radiation to visible light emission derived from the $-NH_2$ passivation[44]. In addition, CCDs have stable fluorescence performance[45]. Therefore, the passivation effect of $-NH_2$ can further modify the surface defects on CDs, making CCDs with better photoluminescence and fluorescence stability, which provides a necessary mean for the application of CCDs in cancer fluorescence imaging.

Yang et al[46] took PEI and folic acid (FA) as raw materials to synthesize CCDs by a one-step hydrothermal carbonization method. The obtained CCDs showed a light yellow color under sunlight, and a bright blue light under 365 nm ultraviolet light. When the wavelength of the excitation light was 370 nm, the corresponding emission wavelength was 452 nm with the maximum fluorescence intensity. The fluorescence emission peak of CCDs had almost no shift under the excitation of 310−410 nm, indicating that the fluorescence emission spectrum was independent of excitation wavelength. It can be illustrated by the fact that the surface state and particle size distribution are changed by $-NH_2$ passivation. In addition, the CCDs can be used for cell fluorescence imaging because of their high photostability. Li et al[47] diluted the CCDs solution prepared from CA and PEI by the hydrothermal method and stored it at room temperature for 6 months. After that, the CCDs solution was irradiated continuously with 360 nm excitation light for 30 min. The fluorescence intensity was kept constant and strong blue light was emitted under the ultraviolet lamp of 365 nm, indicating that the CCDs had good photoluminescence and fluorescence stability (Fig. 4). Wu et al[48] synthesized CCDs from CA, 4-(2-aminoethyl) morpholine and PEI. The introduction of PEI not only enhanced the water solubility of CCDs, but also provided CCDs with $-NH_2$, and the passivation of $-NH_2$ enabled CCDs to maintain fluorescence emission stability under different excitation wavelengths.

3.2.2 Fluorescent quantum yield

The fluorescent quantum yield is an important index to determine the fluorescence intensity of samples. During the fluorescence imaging process of biological organisms, the high fluorescence quantum yield of CCDs can make diseased tissues show bright fluorescence, which is clearly distinguished from normal tissues, thereby obtaining clearer contrast images. Therefore, under light irradiation, the strong fluorescence brightness can provide researchers with useful biological information, which also reflects the great

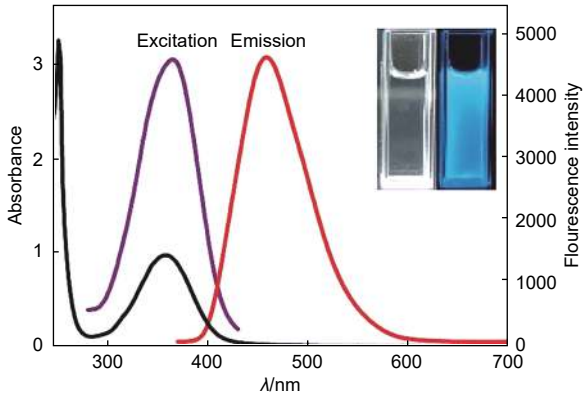

Fig. 4　CCDs show good photoluminescence and fluorescence stability[47] (Reprinted with permission by copyright © 2017 American Chemical Society).

potential of CCDs in biofluorescence imaging.

Yuan et al[49] prepared CCDs with different nitrogen contents (5.5%−19.9%) by controlling the reaction amount of CA and guanidine hydrochloride, and explained that the addition of passivating agent can stabilize the surface of CCDs and promote the generation of energy potential wells, improving the fluorescence quantum yield. They found that there were different functional groups on the surface of CCDs with a low nitrogen content and extremely weak −CO−NH− absorption bands, which led to a low emission efficiency. While the CCDs with a high nitrogen content had a high fluorescence quantum yield and appeared the strong $-NH_2$ absorption band, which was due to the fact that the amino groups enhanced the conjugation degree of conjugated system, and then increased the excited singlet state with the lowest electronic transition probability from ground state to excited state. The more complete passivation can lead to the higher fluorescence quantum yield, which was consistent with the report by Sun et al[50]. Han et al[51] used glucose as the carbon source and PEI as a passivating agent to prepare CCDs, whose fluorescence intensity increased by 300 times compared with that before passivating. Arsalani et al[52] used gelatin and PEG to prepare CCDs with a fluorescence quantum yield of 34%, which had stronger luminescence properties than CDs prepared from a single carbon source of gelatin.

3.2.3　Spectral tunability

The surface state of CCDs is an important factor affecting the emission spectrum. The passivator containing $-NH_2$ can introduce a new surface state for CCDs, while the introduction of different functional groups can lead to the diversity of energy levels and produce the emission trap of absorbed photons, showing the polychromatic excitation[53].

As shown in Fig. 5, Gao et al[54] prepared yellow, blue, and red fluorescent CCDs by coating graphene quantum dots with PEI of different molecular weights. It was found that the energy level of functional groups may be related to their ability to provide electrons. The strong capacity for providing electrons can produce high energy. Therefore, the emission wavelength can be adjusted by PEI passivation on the surface of CCDs. Obviously, the amidation between −COOH and $-NH_2$ played an important role in the coating process, and the CCDs with an adjustable luminescence wavelength can be synthesized by the passivation method, which was consistent with the report by Shahid et al[55].

In addition, Wang et al[56] expressed in their review that the tunability of emission wavelength was related to the passivation degree of $-NH_2$. The lower

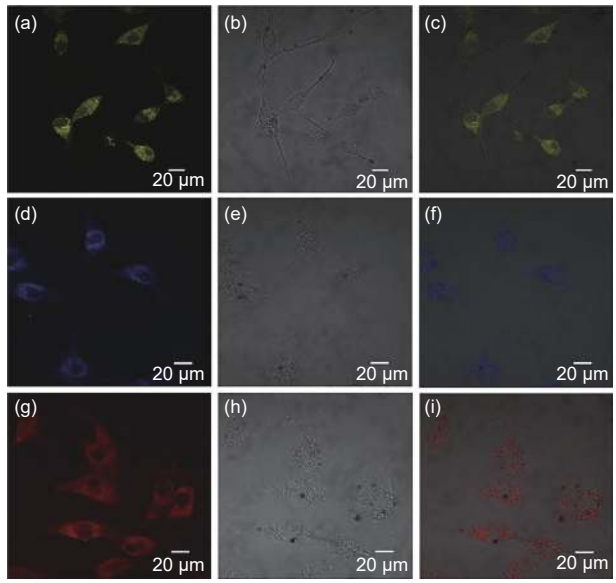

Fig. 5　Confocal fluorescence images of U-87 cells incubated with (a−c) 50 μg mL^{-1} GQDs, (d−f) PEI1800 GQDs, and (g−i) PEI600 GQDs for 18 h. (a), (d), and (g) are the fluorescence images of U-87 cells labeled with these three GQDs, with excitation at 405 nm. (b), (e), and (h) are the bright-field images of U-87 cells. (c), (f), and (i) are the merged images of U-87 cells incubated with three GQDs[54] (Reprinted with permission by copyright © 2017 American Chemical Society).

the passivation degree, the easier it was to exhibit excitation-dependent fluorescence characteristics, which can be achieved by increasing reaction temperature and reducing the amount of $-NH_2$ in raw materials. Therefore, in the process of preparing CCDs, the passivation degree of CDs should be controlled to achieve the emission of multicolor fluorescence and increase the contrast with normal tissue imaging, greatly enriching the selectivity of CCDs in tumor fluorescence imaging, which provided a great possibility for the diagnosis of cancer.

There are different opinions on the light-emitting mechanism of CCDs since it is determined by a combination of multiple factors. However, it is certain that the passivation effect of $-NH_2$ can enhance the fluorescence performance of CCDs, making it a stable and reliable multifunctional fluorescent imaging agent, which can be used for the diagnosis of cancer fluorescence imaging.

3.3 Gene loading capacity

CCDs are positively charged due to the protonation of $-NH_2$ on their surface, so that they can be combined with negatively charged phosphate genes through electrostatic attraction, and then the genes are delivered to cancer cells for transfection[57]. In this process, CCDs play a role in carrying and protecting genes. The branched chain $-NH_2$ is abundant on the surface of CCDs, which has a large surface area and can fully contact with genes. Meanwhile, it can also protect genes from being removed by blood and phagocytic cells, which can greatly improve the gene transfection rate and play a positive role in the gene therapy of cancer.

Wang et al[58] first synthesized CCDs by the microwave method using CA as the carbon source, tryptophan as the nitrogen source and PEI as the passivating agent, and followed by loading CCDs with Survivin siRNA. The combining ability of CCDs with Survivin siRNA was analyzed by agarose gel electrophoresis. The results found that the negatively charged Survivin siRNA neutralized the positive charge of CCDs, causing the electrophoretic migration direction of CCDs to be opposite to that of the control CDs. It was also shown that CCDs can effectively load Survivin siRNA, and then transport to cancer cells to inhibit the proliferation of cancer cells.

Wang et al[59] analyzed Alkyl-PEI passivated synthesized CCDs by agarose gel electrophoresis. When the ratio of N element to P element was greater than five, the CCDs completely blocked the migration of siRNA. When heparin was added, siRNA was released from the CCDs/siRNA complex, indicating that siRNA was loaded on CCDs with strong binding capacity, which can efficiently deliver genes for cancer gene therapy (Fig. 6a). Similarly, Wu et al[60] prepared CCDs/siRNA complexes by mixing CCDs and siRNA with different weight ratios, and used gel electrophoresis technology to study the gene loading capacity of CCDs. The results showed that the siRNA could bind with CCDs when the weight ratio was greater than fifteen, and could be released after treatment with the reductant dithiothreitol (DTT). Wang et al[61] constructed amphiphilic cationic carbon dots (ACCDs) by coupling hydrophobic alkyl epoxides with the surface of CCDs derived from PEI 600 $-NH_2$, which were well soluble in water and organic solvents. By comparing the agarose gel electrophoresis experiments of PEI 600, CCDs and ACCDs at different mass ratios of CCDs and ACCDs to DNA, it was confirmed that CCDs and ACCDs can load DNA well.

4 The basic principles of CCDs used in cancer diagnosis and treatment

Gene-carrying CCDs can pass through the blood circulation to reach cancer tissues. As shown in Fig. 7, The specific recognition sites on their surface can accurately recognize the receptors on cancer cells and bind them together (Step a), then endocyte into the cell to release the gene mediated by specific proteins on the cancer cell membrane (Step b). The released gene corrects the disease-causing gene inside the cancer cells, finally achieving the goal of cancer treatment (Step c). At the same time, CCDs emit bright fluorescence under laser irradiation after entering cancer cells, and fluorescence signals can be monitored

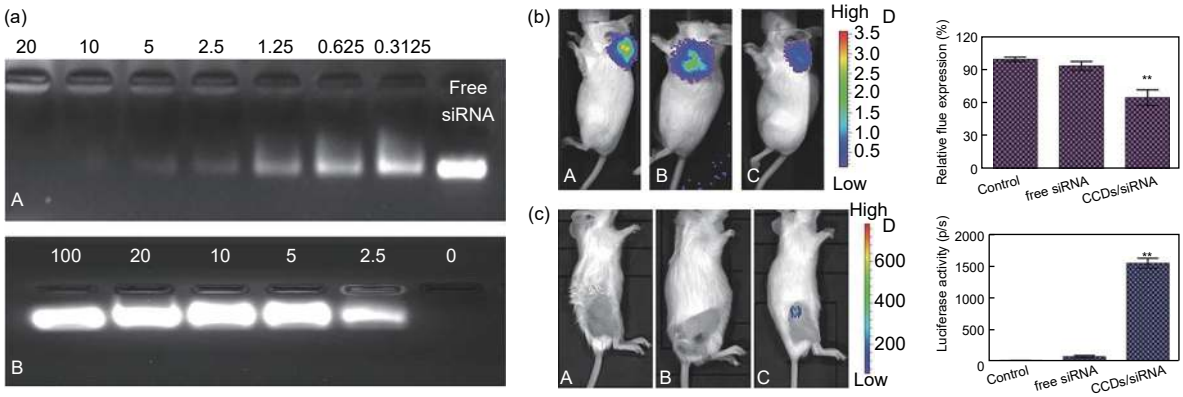

Fig. 6　(a) A) the binding capacity of CCDs and siRNA with different nitrogen/phosphate (N/P) ratios was analyzed by agarose gel electrophoresis; B) the complex formed by CCDs and siRNA with 10 nitrogen/phosphate (N/P) ratio was added to different heparin quantities for heparin decomposition analysis, (b) A−C) in vivo optical imaging, A) control group, B) free siRNA, C) CCDs/siRNA complex, and D) quantitative analysis of luciferase gene expression in fluC-4T1 xenograft after injection of CCDs/siRNA complex and (c) A−C) in vivo optical imaging, and D) quantitative analysis of luciferase gene expression in 4T1 xenograft after injection of CCDs/pDNA complex[59] (Reprinted with permission by copyright © 2014 John Wiley and Sons Ltd).

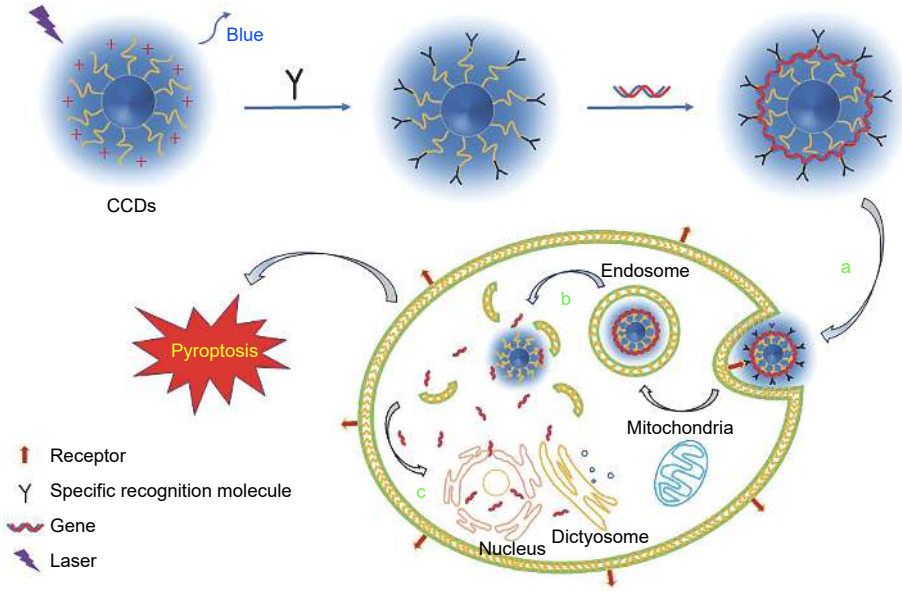

Fig. 7　Schematic diagram of CCDs used in cancer gene therapy.

by fluorescence imaging equipment, which can be used to diagnose cancer.

4.1　CCDs actively targeting cancer cells

Compared with normal cells in the body, cancer cells usually overexpress a series of receptors on their surface, which can bind to specific recognition molecules and induce cell internalization. Specific recognition molecules are like "missiles" and receptors are like "targets"[62−63]. The positively charged $-NH_2$ on the surface of CCDs can not only adsorb specific recognition molecules through electrostatic interaction, but also couple the missile molecules through amide reaction. After CCDs with specific recognition molecules are injected into the body, the missile molecules can automatically track, find and bind to the targets, thereby realizing the active target of CCDs on cancer cells, which is necessary for fluorescent imaging and gene therapy of cancer.

Han et al[51] reported an amide reaction under the activation of EDC/NHS using $-NH_2$ on the surface of CCDs and −COOH in specific recognition molecules as raw materials. Among them, EDC improves the −COOH activity in the specific recognition molecules, and NHS stabilizes the reaction intermediate, so as to enhance the binding ability of −COOH and $-NH_2$ to form −CO−NH−, thereby obtaining CCDs with an

active targeting ability. After CCDs bound with missile molecules and CCDs without missile molecules are incubated with HeLa cells for 1.5 h, separately, the results confirm that the CCDs bound with missile molecules can actively target cancer cells.

4.2 Internalizing into cancer cells

When the receptors on the surface of cancer cells bind to specific recognition molecules on CCDs, they induce CCDs to be transported across the membrane into the cancer cells. At present, most researchers believe that liquid or solute nano-material particles with a diameter of less than 500 nm enter cells mainly through the pinocytosis pathway[64]. The pinocytosis pathway is mainly divided into clathrin-dependent endocytic pathway, caveolin-dependent endocytic pathway, macropinocytosis, microtubule-mediated active transport and cytoskeleton-dependent transport[65]. The entry of nanoparticles into cells is mainly affected by their own physical and chemical properties, such as the size, surface charge, and shape of particles[66]. The particle size of CCDs is generally less than 500 nm and the surface is positively charged, which is mainly transported across the cell membrane through clathrin-dependent endocytosis and caveolin-dependent endocytosis. In addition, the negative charge on the surface of the cell membrane can attract each other with the positive charge on the surface of nanomaterial particles, thereby adsorbing on the surface of the cell membrane so as to promote the nanoparticles to enter the cell.

Zhou et al[67] synthesized CCDs with an average particle size of about 7 nm by a one-step hydrothermal method using sodium alginate as the raw material. After the CCDs and DNA were self-assembled into composite nanoparticles, cell uptake experiments were conducted to investigate how CCDs carried genes into cells. By selecting eight phagocytic inhibitors, after the cells were treated separately, the composite nanoparticles were transfected. The main pathways of the composite nanoparticles into the cells were determined by the intracellular fluorescence intensity at the same time. The results show that the composite nanoparticles are mainly transported into the cell through the combined action of caveolin-dependent endocytosis and clathrin-dependent endocytosis.

4.3 Inhibiting the proliferation of cancer cells

In order to realizing gene therapy, CCDs carry genes into cancer cells and release them in the cytoplasm, such as siRNA, miRNA and P53[68]. Gene silencing therapy has been a research hotspot in recent years. Among them, RNA interference is the most widely applied technology. Under the action of RNA helicase, the siRNA in cell is unzipped into sense strand and antisense strand. The antisense strand can combine with endonuclease, exonuclease and helicase in vivo to form ribosomal complexes with the function of nucleases, namely, RNA-induced silencing complex (RISC). RISC specifically binds to the homologous region of the mRNA expressed by the exogenous gene, and cuts the mRNA at the binding site. The cleavage site is the two ends that are complementary to the antisense strand of the siRNA. The cleaved fragmented mRNA is then degraded, which induces the host cell's degradation response to these mRNAs, and further blocks the expression of cancer genes, finally achieving the effect of silencing the target gene[69].

Shu et al[38] synthesized CCDs by a one-step hydrothermal method using CA and ethylenediamine as carbon sources. The complex formed by CCDs and HER3 siRNA induces cell death by G_0/G_1 phase arrest and apoptosis. Wang et al[58] synthesized CCDs by a microwave method using CA, tryptophan and PEI as raw materials, and then a complex with Survivin siRNA was prepared. After the complex enters the cell, the cell is blocked in the G_1 phase and apoptosis occurs. Wu et al[70] prepared CCDs using CA and ethylenediamine as raw materials by a hydrothermal method, and coupled them with KRAS siRNA to form a complex. The complex showed a good gene knockout efficiency and anti-cancer effect in vitro for treating KRAS mutant non-small cell lung cancer.

5 Application of CCDs in the field of cancer diagnosis and treatment

In the process of integrating cancer diagnosis

with gene therapy, CCDs and genes lack the ability to actively target cancer cells, which greatly compromises the role of CCDs in delivering genes, resulting in poor image quality and low transfection efficiency. At present, the researchers tried to couple CCDs with a specific recognition molecule, including FA, hyaluronic acid (HA) and carcinoembryonic antigen antibody, to specifically bind to receptors on the surface of cancer cells. After that, genes can be selectively and actively delivered to the cancerous tissue for realizing the detection of cancer diagnosis and gene therapy without damaging to normal tissue. Here, the applications of CCDs in three aspects are introduced briefly, including cancer fluorescence imaging, gene therapy, and integration of cancer diagnosis with gene therapy.

5.1 Fluorescence imaging of cancer cells

Cancer cells have the characteristics of rapid growth and unclear separation from surrounding tissues. Traditional inspection methods have a low accuracy for early cancer cell diagnosis and the assessment of goitre extent. With their excellent fluorescence performance and biocompatibility, CCDs specifically bind to tumor cell receptors through targeting molecules to mark cancer cells with a sharp contrast to normal tissues, which is convenient for researchers to distinguish tumor boundaries and early detection of tumors for guiding clinical diagnosis and treatment. The excellent fluorescence performance of CCDs combined with the cancer-specific selection of targeted molecules can present high-quality fluorescence images of cancer tissues, which will provide a new idea for cancer diagnosis.

Du et al[71] covalently coupled green fluorescent CDs obtained by a hydrothermal method with HA, FA and PEI to prepare a dual-ligand modified CCD carrier, which retained the excellent fluorescence characteristics of CDs. The CCDs were used for lung cancer A549 Cells and BEAS-2B normal cells to investigate their imaging ability. The fluorescence in A549 cells was much stronger than that in BEAS-2B cells, which confirmed that the prepared CCDs had the ability of cell-targeted imaging and can be used for the diagnosis of early lung cancer.

Yang et al[46] prepared CCDs using FA and PEI as raw materials by a one-step method, which were incubated with 293T cells and HeLa cells for 4 h. It can be observed that HeLa cells show bright fluorescence, while 293T cells show no fluorescence. This experiment illustrated that the prepared CCDs can be targeted into HeLa cells, which was related to the specific binding of FA on the surface of CCDs and FA receptors on HeLa cells. Moreover, CCDs can emit strong blue light to distinguish between normal cells and HeLa cells, which can be used for early diagnosis of cervical cancer.

5.2 Gene therapy for cancer

At present, PEI is widely used for gene loading due to its high gene transfection rate, which is also regarded as the gold standard for cationic compound gene carriers. Genes can be loaded on the positively charged CCDs by electrostatic interaction to form a complex, and PEI has a “proton sponge effect”, which can effectively release genes in the cytoplasm, thereby achieving efficient gene expression to inhibit or kill cancer cells[72].

A complex was formed by the CCDs prepared by the Alkyl-PEI 2K surface-passivated CDs and siRNA, and then directly injected into the tumor tissue[59]. The luciferase was detected by the imaging system to measure the gene expression level in the xenograft tumor. It was observed that the expression level of luciferase decreased while the other control groups did not substantially decrease, confirming the silencing effect of genes (Fig. 6b). In addition, when the CCDs/luciferase pDNA complex was injected into the tumor tissue, the mice could be seen to emit light, while the control and free pDNA group showed weak luminescence, indicating the successful transfection of pDNA (Fig. 6c). In general, this complex can achieve efficient gene transfection in vivo in animals and can be used for potential gene therapy.

Wu et al[60] prepared CCDs by coupling with PEI to passivate CDs (Fig. 8a), and encapsulated EGFR and cyclin B1 siRNA into CCDs/pooled siRNA and CCDs/single siRNA to treat lung cancer. From Fig. 8b,

it was observed that in the CCD/pooled siRNA group, after treated for 12−48 h, the mRNA gene expression of cyclin B1 and EGFR was inhibited, while the inhibition effect of siRNA without carrier was only short-lived. At the same time, it was also found that CCD/pooled siRNA maintained the gene silencing ability for 48 h, indicating that it had a continuous therapeutic effect. In addition, CCDs/pooled siRNA was used in nude mice to observe the change in tumor size. It was found that in this mode of gene therapy, the intensity of tumor fluorescence imaging in nude mice was reduced compared with before treatment, and the siRNA-loaded complex can be selectively accumulated in lung cancer cells, enhancing the effect of siRNA cancer treatment (Fig. 8c).

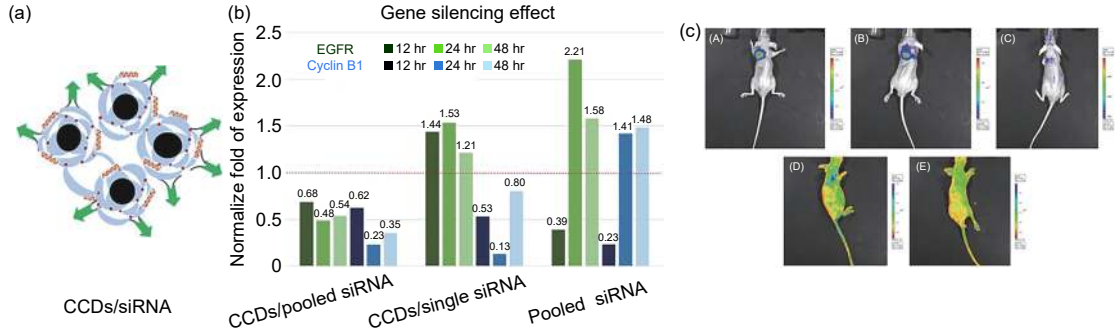

Fig. 8 (a) Diagrammatic drawing of CCDs/siRNA, (b) gene silencing effect of EGFR and Cyclin B1 after CCDs/pooled siRNA, CCDs/single siRNA and pooled siRNA were treated in lung cancer H460 cells for 12, 24 and 48 h, (c) A−C) fluorescence images of lung tumors before and after treatment, B) 7 days after administration, C) 10 days after administration, D) loaded siRNA complexes deposited in the lung area, E) control group[60] (Reprinted with permission by copyright © 2016 Springer Nature).

5.3 Integration of cancer diagnosis with gene therapy

The integration of cancer diagnosis with gene therapy achieved by CCDs can provide real-time image information to guide treatment while cancer is diagnosed, which can avoid diagnosis and treatment in stages, shorten treatment time, and finally realize early detection and treatment of cancer. Therefore, the life quality of cancer patients can be greatly improved.

Yang et al[46] separately co-incubated the normal cells and Hela cells with CCDs, which had excellent fluorescence performance and gene loading capacity. Experimental result found that the Hela cells showed brighter fluorescence than the normal cells. According to the flow cytometry result, CCDs loaded with EGFP can effectively delivery EGFP in Hela cells and transfect the gene EGFP. As shown in Fig. 9, Wang et al[58] also applied the prepared cationic carbon dots carrying siRNA for fluorescence imaging of gastric cancer cells, and verified its ability to inhibit gastric cancer cells. All these results confirm that CCDs have broad application prospects in the integration of cancer fluorescence imaging with gene therapy.

A large amount of $-NH_2$ distributed on the surface of CCDs can not only modify the defects on the surface of CDs, but also make the prepared CCDs show more excellent fluorescence performance, and the introduction of $-NH_2$ leads to the diversity of energy levels, showing the phenomenon of emitting various colors of fluorescence. Moreover, the surface $-NH_2$ has the ability to compress and carry genes, thus acting as a gene carrier. In addition, $-NH_2$ deprotonated and positively charged has a strong cytotoxicity, and a good biocompatibility can be obtained by reasonably controlling the reaction conditions. Grafting tumor-targeting molecules on the surface of CCDs is like installing a radar on gene-carrying CCDs to accurately locate cancer cells. CCDs can be easily found through fluorescence imaging equipment, thereby providing clinicians with more diagnostic information. At the same time, once CCDs enter the cancer cells, the gene release produces a series of reactions, and finally kills the cancer cells, realizing the integration of cancer diagnosis with gene therapy. In the whole process, CCDs play a good carrier and imaging role.

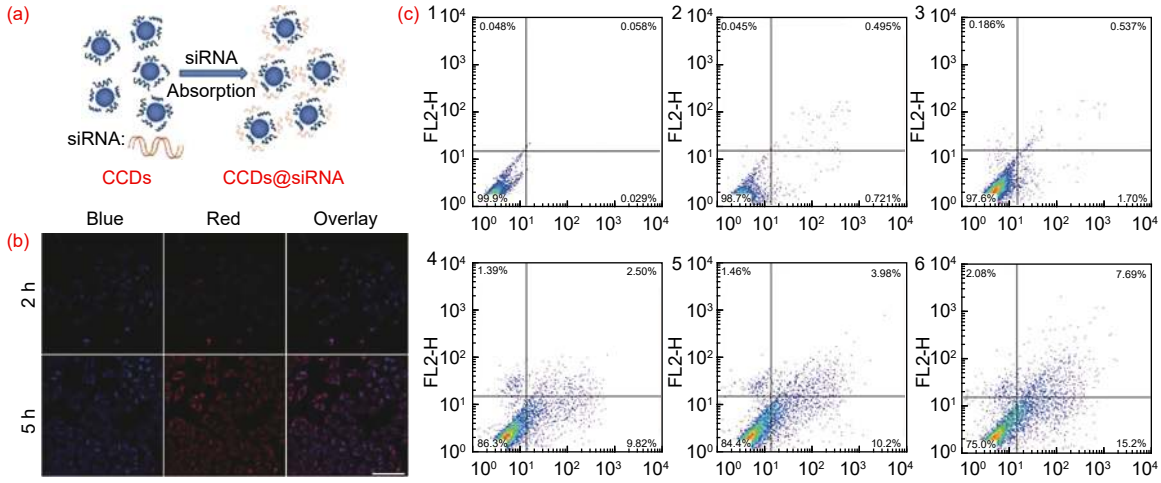

Fig. 9　(a) Schematic diagram of CCDs, (b) confocal laser scanning microscope images of CCDs@siRNA and MGC-803 incubated for 2 h and 5 h, (c) apoptosis analysis of MGC-803 cells treated with CCDs@Survivin siRNA for 48 h. (1−3) are untreated group, sham transfection group and negative control group, (4−6) are Survivin siRNA-3, Survivin siRNA-2 and Survivin siRNA-1[58] (Reprinted with permission by copyright © 2014 BioMed Central).

6　Conclusion and outlook

In summary, CCDs are a new type of nanomaterials prepared by one-step or two-step methods based on cationic compounds. One-step preparation of CCDs is to complete the passivation of CCDs by inheriting $-NH_2$ from the carbon sources to obtain high-density positive charges, and two-step preparation is to use CDs surface passivation modification to obtain excellent performance. A large amount of $-NH_2$ is distributed on the surface of CCDs not only can modify the defects on the surface of CDs, but also make the prepared CCDs show more excellent fluorescence performance, and the introduction of $-NH_2$ leads to the diversity of electronic energy levels, showing the phenomenon of emitting various colors of fluorescence. At the same time, the surface $-NH_2$ has the ability to compress and carry genes, so that CCDs can be used as gene carriers. In addition, $-NH_2$ protonated and positively charged has a strong cytotoxicity, and a good biocompatibility can be obtained by reasonably controlling the reaction conditions. With the excellent biocompatibility, fluorescence performance and gene loading capacity of CCDs, they can be used to realize the dual-mode integration of cancer-targeted fluorescence imaging with gene therapy.

Grafting tumor-targeting molecules on the surface of CCDs is like installing a radar on gene-carrying CCDs to accurately locate cancer cells. CCDs can be easily found through fluorescence imaging equipment, thereby providing clinicians with more diagnostic information. At the same time, once the CCDs enter the cancer cells, the gene release produces a series of reactions, and finally kills the cancer cells, realizing the integration of cancer diagnosis and gene therapy. In the whole process, the loading and protection of genes depend on the positively charged $-NH_2$ on the surface of CCDs. The fluorescent properties of CCDs make it easy for fluorescence imaging of cancer cells. Coupled with their good biocompatibility, CCDs show good prospects in the field of cancer diagnosis and gene therapy. However, the research of CCDs as gene carriers is in its infancy, there are still many problems to be overcome, as follows.

(1) The preparation of CCDs depends on the preparation process of CDs. These processes have some problems such as low yield and poor size controllability. To find a new preparation method with large-scale, simple and easy control of properties is the major way to expand the application of CCDs.

(2) CCDs are mainly synthesized by a one-step method with amine-containing compounds and other carbon containing compounds as raw materials and a two-step method with self-assembly modification of the cationic compounds on the surface of CDs. It is not easy to determine the optimal gene loading capacity by adjusting the exact ratio of raw materials. In

addition, a high positive charge density brings a higher cytotoxicity for cells. Therefore, the investigation on the precise ratio of raw materials and precise control of reaction conditions is a way to improve the preparation and application of CCDs.

(3) There are few studies on the endocytosis mechanism, in vivo imaging, metabolic pathways, fluorescence emission mechanism and the relationship between CCDs and organelles. Further research may help to deepen the understanding of CCDs for improving fluorescence imaging and gene loading conveying capacity.

In short, the integration of cancer diagnosis and gene therapy has developed rapidly in recent years. Compared with a single mode diagnosis or gene therapy, it has presented great advantages. It not only can save patients' turnaround time, but also optimize medical resources. The key to achieve the integration of cancer diagnosis with gene therapy lies in the rational preparation of “vectors”, integrating imaging agents and genes. The emergence of CCDs is expected to promote the rapid development of cancer diagnosis and gene therapy technologies, which provides a new hope for humans to overcome cancer.

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阳离子碳点在癌症诊断和基因治疗一体化中的研究进展

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摘 要: 阳离子碳点 (CCDs) 是表面带正电荷的一类碳点 (CDs), 可通过 CDs 和含氨基 (—NH_2) 的阳离子化合物经“一步法”或“两步法”制备得到, 既保留了 CDs 良好的荧光性能、低毒性及生物相容性, 又提高了基因的负载输送能力及细胞摄取能力, 使得 CCDs 在癌症靶向荧光成像和基因治疗领域中有重要的应用价值。本文综述了 CCDs 的制备方法和优异性能, 说明 CCDs 可作为显像剂及基因的良好靶向载体; 此外, 还介绍了 CCDs 用于癌症诊治的基本原理及在癌症诊断和基因治疗方面的应用, 并提出目前 CCDs 面临的问题及未来的发展趋势。

关键词: 阳离子碳点; 基因治疗; 荧光成像; 癌症诊治一体化

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