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Review

Shedding light on gene therapy: Carbon dots for the minimally invasive image-guided delivery of plasmids and noncoding RNAs - A review

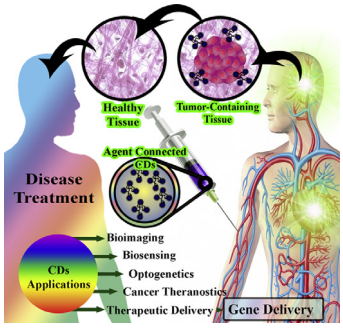
Reza Mohammadinejad [a], Arezoo Dadashzadeh [b], Saeid Moghassemi [b], Milad Ashrafizadeh [c], Ali Dehshahri [d], Abbas Pardakhty [a], Hosseinali Sassan [e], Seyed-Mojtaba Sohrevardi [f,*], Ali Mandegary [g,*]

[a] Pharmaceutics Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran
[b] Department of Biomedical Engineering, Amirkabir University of Technology, Tehran, Iran
[c] Department of Basic Science, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran
[d] Department of Pharmaceutical Biotechnology, School of Pharmacy, P.O. Box: 71345-1583, Shiraz University of Medical Sciences, Shiraz, Iran
[e] Department of Biology, Faculty of Sciences, Shahid Bahonar University, Kerman, Iran
[f] Pharmaceutical Sciences Research Center, Faculty of Pharmacy, Shahid Sadoughi University of Medical Silences, Yazd, Iran
[g] Neuroscience Research Center, Institute of Neuropharmacology, and Department of Toxicology & Pharmacology, School of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran

HIGHLIGHTS

- Bioimaging and gene therapy are of interest for cancer theranostics.
- Carbon dots (CDs) possess superior physicochemical properties.
- CDs can be used as imaging-trackable gene nanocarriers.
- CDs with high transfection efficiency have been applied for condensing plasmids.
- Biocompatible CDs presented no distinct adverse impacts at high concentration.

GRAPHICAL ABSTRACT



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ABSTRACT

Recently, carbon dots (CDs) have attracted great attention due to their superior properties, such as biocompatibility, fluorescence, high quantum yield, and uniform distribution. These characteristics make CDs interesting for bioimaging, therapeutic delivery, optogenetics, and theranostics. Photoluminescence (PL) properties enable CDs to act as imaging-trackable gene nanocarriers, while cationic CDs with high transfection efficiency have been applied for plasmid DNA and siRNA delivery. In this review, we have highlighted the precursors, structure and properties of positively charged CDs to demonstrate the various applications of these materials for nucleic acid delivery. Additionally, the potential of CDs as trackable gene delivery systems has been discussed. Although there are several reports on cellular and animal approaches to investigating the potential clinical applications of these nanomaterials, further systematic multidisciplinary approaches are required to examine the pharmacokinetic and biodistribution patterns of CDs for potential clinical applications.

Introduction

Gene therapy may improve the health of patients with a variety of inherited and acquired human conditions including cancer,

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* Corresponding authors.
E-mail addresses: smsohrevardi@ssu.ac.ir (S.-M. Sohrevardi), alimandegary@kmu.ac.ir (A. Mandegary).

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diabetes, cardiovascular diseases, and mental disorders. The use of nucleic acids including pDNA, mRNA, and noncoding RNAs as gene therapy modalities is rapidly advancing into clinical practice. Recently, clustered regularly interspaced short palindromic repeat (CRISPR)–CRISPR associated 9 (Cas9) nucleases mediated by a specific short guide RNA were shown to be effective for genome editing [1]. Many additional gene therapy systems are currently under clinical trials or preclinical evaluation [2].

The success of gene therapy relies on the generation of a carrier that can effectively and selectively deliver a gene to target cells with low toxicity. Because of their favourable properties, including ease of production and chemical characterization, large packaging capacity, lack of immunogenicity, and potential for tissue specificity, nanoparticles (NPs) have received significant attention as non-viral gene transfer vectors, providing an alternative to the popular viral vectors. Many types of nanomaterials, such as polymeric NPs [3,4], noble/transition metal-based NPs, carbon nanomaterials, and biological nanostructures, have been investigated as non-viral nucleic acid nanocarriers [5–9]. Because many nanovectors that transfect cells *in vitro* fail to function or have high toxicity *in vivo*, the gene delivery efficiency of the non-viral methods remains a key barrier to clinical use [10].

Recently, CDs have been identified as a potential material for nanomedicine applications [11]. Highly fluorescent surface passivated/functionalized CDs with good stability in physiological environments have been easily fabricated for trackable cancer-targeted therapy. Biocompatible CDs are minimally invasive NPs and are excreted from the body in a reasonable period of time without obvious side effects. Small-scale CDs possessing low toxicity, high quantum yield, low photobleaching, good water solubility, easy surface modification, and chemical stability are emerging nanocarriers for gene delivery applications. Liu et al. in 2012 [12] and Wang et al. in 2014 [13] reported for the first time that CDs could serve as safe and efficient imaging-trackable nanocarriers for *in vitro* and *in vivo* gene delivery. There are various studies on the potential use of CDs for the delivery of pDNA [14]. Recently, CDs were also applied to condensing small interfering RNA (siRNA).

Photoluminescence features of carbon dots

CDs are a novel subset of carbon nanoallotropes that have, due to their significant PL properties and excellent photostability, become a potential material for biomedical applications [15]. The particle size, shape, concentration, composition, and internal structure can affect the fluorescence emission spectra of the CDs. The role of precursors in the emission maxima of the CDs was investigated, and emission maxima of λ = 435, 535, and 604 nm were calculated for m-CDs, o-CDs, and p-CDs from phenylenediamines (three isomers: m-phenylenediamine, o-phenylenediamine, and p-phenylenediamine), respectively [16]. The maximum fluorescence emission wavelengths of CDs also vary greatly, from the visible to the near-infrared region, in various solvent species.

There are various methods by which surface alteration can elevate the PL of CDs, such as hydrothermal carbonization [17] and microwave synthesis [18]. However, among them, the surface passivation method is the most beneficial and most common, because higher PL activity of CDs can be obtained by better surface passivation [19,20]. In the surface passivation process, the inactivation of surface defects of CDs could prevent nonirradiative emissions. Additionally, quantum yields as high as 93% can be achieved by a single-step process without any post-synthetic treatments [21]. It has also been shown that the concentration of N and the proportion of C—N and C═N can improve the PL [22]. Polyethylene glycol (PEG) [23,24] and polyethyleneimine (PEI) [12,25] are the most commonly used surface passivating agents. Meanwhile, heteroatom-doped CDs have been prepared for the regulation of their intrinsic features (Fig. 1).

For example, nitrogen-doped CDs demonstrated superior luminescence performance and excellent electrochemical function. Upconversion and IR fluorescent heteroatom-doped CDs are particularly desirable for live deep-tissue imaging, diagnosis and therapy [26].

Biocompatibility of carbon dots

Toxicity concerns continue to be one of the largest obstacles to the clinical translation of NPs [27]. Semiconductor QDs, which are fluorescent NPs, are used for different applications, particularly for bioimaging [28]. Cadmium-containing QDs are more beneficial than conventional organic dyes, but the toxicity of QDs is their most challenging drawback [29,30]. These QDs are toxic even at low levels and can accumulate in organs and tissues [31]. Acute toxicity and prothrombotic impacts have been demonstrated as drawbacks of QDs in mice; however, biocompatibility is one of the most important properties for bench-to-bedside translation of QDs [32]. The development of photoluminescent nanoprobes that do not contain heavy metals was introduced in the pursuit of biocompatibility by Xu and coworkers in 2004 [33]. Importantly they are not toxic to the environment and have high solubility in water with long-lasting colloidal durability, which makes them good substitutions for semiconductor QDs (Fig. 2) [34].

Due to the promise of the applications of CDs in nanomedicine, concerns about their safety have drawn increasing attention recently [35], and extensive studies on the cytotoxicity of luminescent CDs have been reported. *In vitro* studies have demonstrated that CDs are usually safe for numerous cell lines. Several *in vivo* studies showed that CDs could be found in various organs, but the amount of accumulation was remarkably low. No meaningful toxicity, clinical symptoms, death or even remarkable body weight drops have been reported [36].

Furthermore, histopathological investigations of treated mice presented no obvious impairment at the high CD concentrations required for PL bioimaging; the structures of the organs from the treated mice were ordinary, almost identical to those of the control group. Biochemical analysis showed no significant alterations in most of the measured biochemical parameters in the tissues and serum, except for a slight reduction in the albumin level in serum, as well as AChE activity in the liver and kidneys. Recently, Hong et al. [35] provided deep insights into the toxicity of CDs *in vivo* by ^{1}H NMR-based metabolomics. They reported that CDs affect the immune system, cell membranes and normal liver clearance. Due to fast, high uptake in the reticuloendothelial system, NPs with large particle sizes (>10 nm) have provoked increased long-term toxicity concerns. Accordingly, biodegradable larger NPs and renal-clearable ultra-small NPs have been explored for biologically safe theranostic nanomedicine [27].

CDs are efficiently and rapidly excreted from the body after intravenous (iv), intramuscular (im), and subcutaneous (sc) injection [37]. The injection route affects the rate of blood and urine clearance, the biodistribution of CDs in major organs and tissues, and tumour uptake over time. The clearance rate of CDs is ranked as intravenous (iv) > intramuscular (im) > subcutaneous (sc). In clinical applications, various injection routes can be applied for various purposes, such as tumour targeting, long circulation, or ease of use by the physician. These characteristics make CD-based nanoprobes as viable candidates for clinical translation [38].

Recently, Licciardello et al. [39] reported that the behaviour of CDs is dictated by their surface features. For example, with biocompatible PEG conjugates, gadolinium metallofullerene nanocrystalline (GFNCs)–CDs–PEG nanocarbon becomes highly

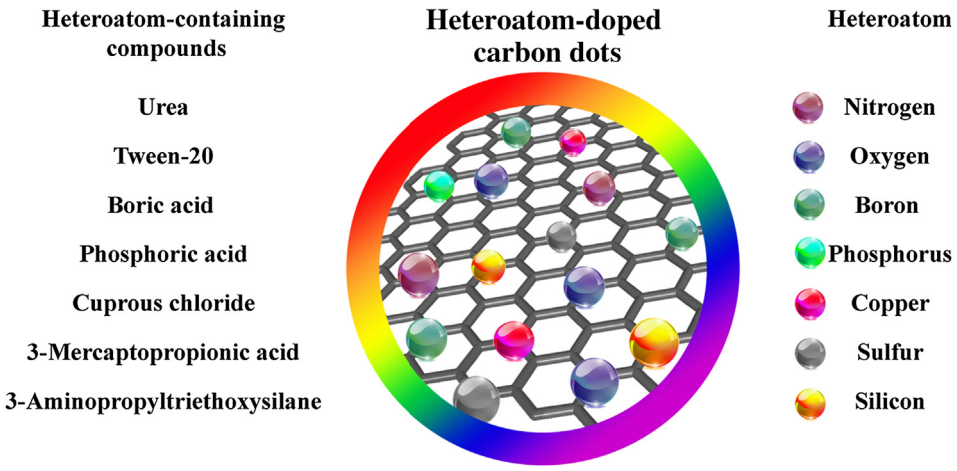

Fig. 1. Heteroatom-containing compounds as precursors to produce heteroatom-doped CDs.

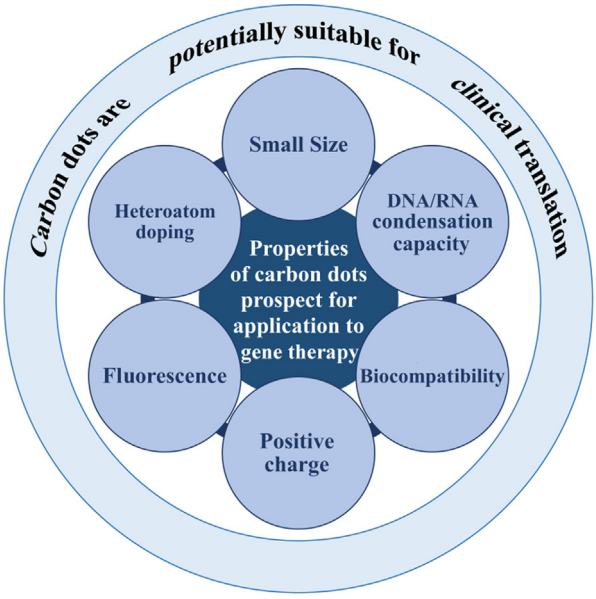

Fig. 2. CDs are suitable nanocarriers for nucleic acid delivery.

stable in physiological environments and is excreted from the body in a reasonable period of time without obvious side effects [40].

Preparation methods for carbon dots

Spherical-like CDs with a size of 10 nm are easily produced using many precursors, such as natural and synthetic molecules and polymers [41]. "Top-down" and "bottom-up" methods (Table 1) have been used to synthesize three types of CDs, namely, graphene quantum dots (GQDs), carbon nanodots (CNDs), and polymer dots (PDs) [42].

Hydrothermal carbonization, pyrolysis or thermal decomposition, and microwave irradiation are among the preparation methodologies (Fig. 3). Recently, Meng et al. [55] produced a high level of CDs with an inexpensive method that does not require exterior warming or supplementary energy input.

Precursors and surface passivation agents to prepare cationic carbon dots

The structure of nucleic acid materials including DNA, consists of negatively charged phosphate groups. The electrostatic interaction between nucleic acids and positively charged materials such as cationic compounds results in the formation of particles in sizes ranging from nanometres to micrometres. These positively charged structures can interact with the negatively charged components of the cell membrane, including proteoglycans. The interaction between particles and cell membranes leads to adsorptive endocytosis, resulting in the formation of endosomes [56]. Because of the abundance of amines on the surface of the materials used for the formation of these positively charged structures, the proton sponge effect leads to early escape of the particles from endo/lysosomal vesicles before enzyme degradation begins inside the compartments. In other words, the nucleic acid materials may be released into the cytosol prior to the activation of degrading enzymes. These properties make the particles appropriate carriers for transferring various nucleic acid materials into different cells [57].

Positively charged compounds or polycations have been used for the synthesis and surface passivation of cationic CDs. Due to the positive fragments on the surface of the CDs, these structures are also able to interact with DNA to create a complex through electrostatic attraction. Significant attention has been directed to synthetic polymers containing amines, including PEI, chitosan, poly-L-lysine (PLL), and poly(amidoamine) (PAMAM) (Fig. 4).

Table 1
Methodologies for the preparation of CDs.

Strategies	Methods	Advantages	Disadvantages	Refs
Bottom-up	Microwave synthesis	Easily controllable size, uniform size distribution, short reaction time	High energy cost	[43–46]
	Thermal decomposition	Large-scale generation, low cost, easy operation	Broad size distribution	[47,48]
	Hydrothermal treatment	Lack of toxicity, low cost, superior quantum efficiency	Low yield	[49,50]
Top-down	Laser ablation	Morphology and size control	High cost, sophisticated process	[51]
	Electrochemical oxidation	High purity and yield, size control	Sophisticated process	[52,53]
	Chemical oxidation	Large-scale generation, easy process with simple tools	Broad size distribution	[48]
	Ultrasonic treatment	Easy process	High energy cost	[54]

Fig. 3. Devices to produce CDs.

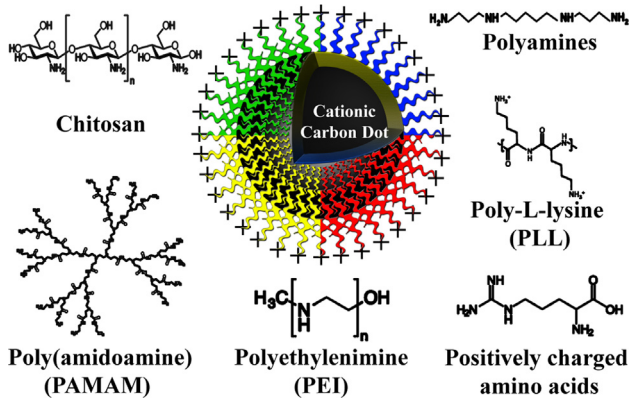

Fig. 4. Cationic materials to produce positively charged CDs.

Polyethylenimine

Several polycationic compounds have been used for gene and drug delivery. PEI can be considered the gold standard for non-viral gene delivery by polycations [58]. The high positive charge density on the PEI surface enables the molecule to interact with negatively charged macromolecules such as pDNA or siRNA [59]. The polymer contains primary, secondary and tertiary amines. Since the primary and secondary amines are oriented towards the exterior of the molecule, it could be postulated that these amines are primarily responsible for nucleic acid condensation, whereas the tertiary amines oriented towards the interior of the molecule are primarily responsible for protonation in acidic environments (e.g., endo/lysosomal vesicles) and induction of the proton sponge effect. In other words, the primary and secondary amines condense nucleic acids and form polyplexes, and the tertiary amines induce early escape from endosomes [60,61]. The cooperative behaviour of various amines in PEI molecules makes the polymer a powerful candidate for gene delivery [62]. The considerable transfection efficiency of PEI is dependent on the molecular weight and charge density of the polymer; however these factors are also the major causes of its remarkable cytotoxicity. Therefore, charge modulation could be a promising strategy for improved viability and transfection efficiency [63]. One of the best recognized modifications of the PEI structure is the conjugation of a hydrophilic moiety such as PEG to modulate the charge and improve the biophysical properties of the polymer, as well as to ameliorate its cytotoxic effects [64].

Poly(amido amine)

Poly (amidoamine) (PAMAM) is a dendrimer with a highly branched spherical structure, well-defined diameter, low polydispersity, and several amino groups in its structure [65]. PAMAM is extensively applied for biosensing, drug and gene delivery as well as imaging [66]. Divergent and convergent methods or a mixture of these strategies can be applied for PAMAM synthesis [67]. The ability of PAMAM to pass through the cell membrane makes it a suitable drug delivery vehicle [68]. Dendrimers have been reported to enter Caco-2 cell monolayers via the paracellular pathway in an energy-dependent manner; however their intracellular destination is not clear. The manner of the cell internalization of PAMAMs is influenced by their size and surface charge. Furthermore, PAMAM dendrimers demonstrated high efficiency in transferring genetic materials into different cells and organs [69].

Chitosan

Chitosan, a polymer with cationic features has shown a remarkable ability to act as a gene delivery vector. Protonation of the primary amines of chitosan at low pH leads to interaction with negatively charged macromolecules [70]. The application of chitosan as a non-viral gene delivery system for plasmid transfer was first introduced in 1995 [71]. Additionally, various studies have led to the successful application of chitosan and its derivatives for DNA delivery. Furthermore, chitosan has been applied to the condensation of siRNA since 2006 [72]. Recently, cystic fibrosis cells have been treated with chitosan/miRNA complexes [73]. Various attempts have been made to demonstrate the impact of the degree of deacetylation and the level of polymerization on the biophysical properties of chitosan-based systems and their biological action. In addition, several studies show relationships of the salt form and pH to the pDNA delivery capability and intracellular trafficking pathways [74].

Ethylenediamine

Ethylenediamine (EDA) (—NCH_2CH_2N—) has been widely used in metal complexes. These complexes are considered significant anticancer compounds due to their redox chemistry and simple modification. Metal complexes containing EDA can stimulate cytotoxic function in various cancer cell lines [75]. However, it is necessary to develop novel EDA-type ligands as chemotherapeutic agents. In addition, EDA-type ligands have shown antimicrobial, antifungal, antibacterial, antituberculosis, antimalarial, antileish-

manial and antihistamine activities [76], and EDA has been applied as a surface passivation compound to synthesize N-doped CDs [77].

Polyamines

Polyamines contain at least three amino groups. Low-molecular-weight linear polyamines are found in biological systems. The most studied natural polyamines are spermidine and spermine, which are structurally and biosynthetically related to the diamines putrescine and cadaverine. Polyamines have been used for the synthesis of supercationic (ζ-potential ca. +45 mV) CDs and are rich in nitrogen. CDs produced by the direct pyrolysis of spermidine (Spd) powder exhibit much higher solubility and yield than those from putrescine and spermine [78].

Positively charged amino acids

It has been reported that cationic amino acids including arginine and lysine, can be conjugated to PAMAM dendrimers to ameliorate the transfection ability of unmodified dendrimers. The addition of arginine and lysine to the PAMAMs ameliorated the DNA condensation ability via increased charge density on the surface of dendrimers [79]. Furthermore, the guanidium group of arginine has a positive charge and demonstrates superior interaction with the phosphate in DNA to that of ammonium. In addition, the guanidium group of arginine has a remarkable affinity for cell membranes via hydrogen bonding and ionic pairing. Due to these features, dendrimers modified with arginine and lysine have superior properties for use as carriers for pDNA and siRNA [80,81]. Histidine modification can also be used to ameliorate the transfection ability of cationic PAMAM dendrimers. The histidine-modified dendrimers are serum resistant due to the static nature of the imidazole group; in addition, the conjugation of histidine into dendrimers improves the pH-buffering capacity of the dendrimers. In addition, guanidium and imidazolium-modified dendrimers demonstrate elevated transfection ability [82]. Another reason for the increased transfection ability of cationic polymers is the generation of an equilibrium between the charged and hydrophobic content of the polymer. Hydrophobic amino acids such as phenylalanine and leucine have been shown to increase the transfection ability. In addition, these conjugates of polycationic polymers transfer siRNA more efficiently than the unmodified parent polymers [83–85]. A mixture of arginine, histidine and phenylalanine was also shown to have an increased impact on the gene delivery efficacy of PAMAM dendrimers [86].

Applications of carbon dots as trackable gene delivery systems

Bioimaging and gene therapy are interesting for the diagnosis and therapy of various diseases, but there are few approaches that achieve both purposes at the same time. In recent years, multifunctional CDs, such as fluorescent nanoprobes have been successfully used for *in vitro* and *in vivo* intracellular imaging and cancer theranostics. Transferrin-[87], RGD peptide-[88], folic acid (FA)-[89], and hyaluronic acid-conjugated [90] CDs have been applied as fluorescent probes for accurate tumour diagnosis and targeting therapy [91,92]. Zhang et al. [93] reported that after the conjugation of FA to green luminescent CDs, the photostable FA-CDs selectively entered HepG2 cancer cells via folate receptor (FR)-mediated endocytosis. The FA-CDs could accurately recognize FR-positive cancer cells in various cell mixtures [94]. Recently, Li et al. [95] demonstrated visualized tumour therapy by emancipating stable FA-modified N-doped CDs (FN-CDs) from autophagy vesicles. The method achieved a strong therapeutic effect *in vitro* and *in vivo*. The combination of FN-CDs and autophagy inhibitors caused rapid inhibition of tumour cell growth (within 24 h) and efficient killing effects (killing rate: 63. 63–76. 19% in 4 d) in up to 26 different tumour cell lines. Animal model experiments showed that the 30-d survival rate of the method was up to 98%, much higher than that of traditional chemotherapy (68%). Accordingly, stable surface-modified CDs have been used for noninvasive real-time image-guided targeting tumour therapy.

In addition, the encapsulation of CDs with liposomal formulations can be used for tumour angiogenesis imaging [96]. Recently, Wu et al. [97] developed CDs to encapsulate siRNA for imaging-guided lung cancer therapy. The theranostic CDs absorb at 360 nm and emit at 460 nm, the wavelength of blue light. In the diagnostic modality of the theranostic CDs, the highest PL appeared at 460 nm, and in the therapeutic segment, apoptotic cell death occurred.

CDs have great potential for use in live-cell and *in vivo* bioimaging due to their attractive luminescence properties and resistance to photobleaching. However, CDs mostly show intense emissions at short blue or green wavelengths, and the inefficient excitation and emission of CDs in both near-infrared (NIR-I and NIR-II) windows remain an issue [98,99,100]. Solving this problem would yield a significant improvement in the tissue-penetration depth for *in vivo* bioimaging with CDs [101,102]. The surface treatment of CDs with molecules rich in sulfoxide/carbonyl groups can thus be considered a universal method for developing NIR imaging agents and realizing CD applications in *in vivo* NIR fluorescence imaging. Recently, Li et al. [101] provided a rational design approach to develop surface-modified CDs with poly(vinylpyrrolidone) in aqueous solution that was successfully applied for the *in vivo* NIR fluorescence imaging of the stomach of a living mouse. The poly(vinylpyrrolidone) groups, which were bound to the outer layers and the edges of the CDs, influence the optical bandgap and promote electron transitions under NIR excitation. The study represented the realization of both NIR-I excitation and emission and the two-photon- and three-photon-induced fluorescence of CDs excited in an NIR-II window for clinical applications of CD-based NIR imaging agents. In another study, Lu et al. [102] fabricated NIR-emissive polymer CNDs, which had a uniform dispersion and an average diameter of ≈7.8 nm with two-photon fluorescence. They demonstrated *in vivo* bioimaging based on low-cost, biocompatible CDs.

In the field of gene therapy, genetic materials or silencing nucleic acids (e.g., siRNA) are introduced into cells to affect specific signalling pathways and certain targets, resulting in the slowed or reversed progression of disease. The basic concept of gene therapy is the transfer of genetic material to a patient's cellular nucleus to increase gene expression or produce a target protein by RNA transfection [103]. Diseases such as cancer, Parkinson's disease, AIDS and cardiovascular ailments can be treated by gene therapy. There are two gene carrier classifications: viral and non-viral vectors. It is more difficult for non-viral vectors to diffuse in the targeted tissue, due to the lack of anterograde and retrograde transportation. The greatest challenge in overcoming the concern of diffusion for gene delivery is the promotion of intracellular transport ability. It is important that the vectors used in gene therapy have low toxicity, high stability and a prolonged circulation time in the bloodstream [104,105].

Biocompatibility, inexpensive fabrication techniques, the ability to bind to inorganic and organic molecules, low toxicity, nano size for *in vivo* cellular uptake, high water solubility and different routes of administration (nasal, oral, parental and pulmonary) make CDs better choices for gene delivery than other non-viral vectors. Cationic polymers such as PEI have been used in the gene delivery field but have caused cell necrosis due to the aggregation of PEI clusters in cell membranes and undesirable effects on blood components. Multifunctional nanodelivery systems can respond to

several exogenous or endogenous stimuli, such as pH, temperature, redox conditions, and magnetic or ultrasound fields [106]. Stimuli-triggered release is a promising approach for nucleic acid delivery with spatiotemporal and dosage control [107]. Recently, Wong et al. [108] synthesized stimuli-responsive NPs composed of cationic β-cyclodextrin-modified polyethyleneimine (CDP), tetronic polyrotaxane end-capped with adamantane (Tet-PRX-Ad) and CD-RGD to package microRNA (miRNA) and pDNA. The self-assembled NPs disassembled at endosomal pH, allowing the release of αCD molecules to induce endosomal rupture and render the plasmids available for nuclear transport.

CDs have desirable properties such as low toxicity, chemical stability, biocompatibility, easy surface modification and good water solubility [109–111], low photobleaching and the potential for widespread applications in bioimaging fields that make them appropriate nanomaterials for *in vivo* imaging compared to other NPs [3]. CDs, which usually have a size < 10 nm, have been shown to have superior properties and to qualify as a functional nanomaterial. In comparison with other fluorescent carbon NPs, they are superior due to their quantum yield, aqueous solubility, facile synthesis, physicochemical properties and photochemical stability [112]. PEI and amine compounds such as EDA, spermine, and arginine have been used for the surface coating of CDs. CDs with cationic charge can efficiently transfect the therapeutic plasmid into cells. Because of their positive charge, cationic polymers can bind to negatively charged DNA and facilitate intracellular transfection. Citric acid- and PEI-derived CDs containing survivin siRNA demonstrated an inhibitory effect on the development of human gastric cancer cells MGC-803 and acted as an imaging agent [13]. In addition, higher gene expression ability and lower cytotoxicity have been reported for CDs containing vectors than for the polymer alone [12]. CDs exhibit fluorescent properties and gene delivery functions that can greatly benefit gene transfection procedures (Table 2).

CDs can also be used as carriers for drugs, mostly anticancer drugs such as doxorubicin that can conjugate to CDs derived from citric acid and urea via carboxyl groups. Due to the photoluminescent property of CDs, drug release at the tumour site can be monitored [112].

Plasmid delivery

One of the best strategies for gene delivery is the use of CD-compressed pDNA, which can enhance gene transfection up to 104-fold over naked DNA delivery [25]. Generally, in a simple method for preparing pDNA loaded CDs, plasmids are amplified and purified before vector loading, and the CDs/pDNA are prepared by pipetting two separated CDs and a pDNA solution (at defined concentrations) and then incubating the mixture [114].

Chen et al. [113] studied the use of gene therapy for ectodermal mesenchymal stem cells with CDs. The CDs were derived from porphyra polysaccharide, coated with EDA (acting as a passivation agent) and finally loaded with the optimal combination of transcription factors Ascl1 and Brn2. The results showed more efficient neuronal differentiation of the EMSCs with CDs/pDNA NPs than with the all-trans retinoic acid-containing induction medium. Cao et al. [114] successfully used CDs for the delivery of plasmid SOX9 (pSOX9) into mouse embryonic fibroblast cells. They surveyed the toxicity of CDs/pSOX9 with the MTT assay and its immunogenicity by the intravenous (IV) injection of mice, and the results demonstrated the biosecurity and low toxicity of CDs. In addition, an *in vitro* study indicated that CDs/pSOX9 had high gene transfection efficiency and enabled the intracellular tracking of the delivered molecules. Furthermore, photoluminescent cationic CDs provided dual functions, self-imaging and effective non-viral gene delivery. Ghafary et al. [122] synthesized CDs/MPG-2H1 with DNA loading to obtain green and red emission, endosomal escape and targeting of the cellular nucleus. The CD/MPG-2H1s increased the plasmid-refuging firefly luciferase gene internalization of HEK 293T cells, showing that this carrier has high potential for increasing nuclear internalization increases. Another study by Zhou et al. [119] synthesized CDs using alginate and showed the use of CDs for the delivery of the plasmid TGF-b1 (pTGF-b1) into 3T6 cells. The results of this study showed that CDs had a strong capacity to condense pDNA, with suitable biocompatibility, low toxicity and high transfection efficiency. More examples of pDNA delivery by CDs are shown in Fig. 5.

siRNA delivery

Noncoding RNAs (ncRNAs) refer to RNA molecules that do not encode a protein. However, ncRNAs, including miRNA, intronic RNA, repetitive RNA and long noncoding RNA, can modulate genome transcriptional output [123–125]. Endogenous noncoding RNAs (miRNAs) and chemically synthesized siRNAs have shown great potential for use in nucleic acid therapeutics [91]. siRNA is a type of double-stranded RNA (dsRNA) molecule 20–25 base pairs in length that has specific RNAi-triggering actions, such as cleaving the mRNA before translation [13,126].

The delivery of siRNA is one of the best therapeutic candidates for treating incurable diseases and has remained an interesting issue for gene delivery researchers due to its high efficiency of intracellular delivery [127]. siRNA is a rigid molecule due to the packing of strong cationic agents and is thus difficult to condense. Obstacles to the use of siRNA macromolecules include difficulty in traversing the membrane and in escaping from endosomes into the cytosol. To overcome this problem, some cationic lipid- or polymer-based transfection reagents have been investigated in *in vitro* experiments. The nanocarriers used to deliver siRNA therapeutics can be modified with specific ligands (i.e., FA, hyaluronic acid) to deliver therapeutic agents to specific cells [95]. Wang, et al. [13] demonstrated that siRNA molecules can interact with the Alkyl/PEI2k/CDs surface. They studied the treatment of gastric cancer cells MCG-803 using CDs/siRNA and determined characteristics such as the efficacy of gene transfection, siRNA delivery into cells, and the influence of CDs/siRNA on biological processes. The results indicated that siRNA can attach to the surface of CDS and that the use of CDs/siRNA notably enhanced the gene delivery efficiency. Additionally, Wu et al. [97] investigated the treatment of lung cancer by folate-conjugated reducible PEI-passivated CD (fc/rPEI/CD) NPs with EGFR and cyclin B1 as two types of siRNA. These fc/rPEI/CDs/siRNA NPs can accumulate in cancer cells and improve the gene silencing and cancer treatment effects of the siRNA. Moreover, Dong et al. [128] studied poly(L-lactide)(PLA) and PEG-grafted GQDs as nanocomposites for simultaneous gene delivery usage and intracellular miRNA bioimaging. The results showed that the functionalization of GQDs with PEG and PLA provides the nanocomposite with super-physiological stability, with low cytotoxicity induced by different concentrations (14, 28, 70, 140 μg/mL). The functionalized GQD nanocomposite had stable PL over a broad pH range. These results suggest that this nanocomposite has high potential in biomedical use for diagnosis and therapy.

Pierrat et al. [118] studied cationic CDs/siRNA and its biocompatibility and performance for *in vivo* transfection by intranasal administration into mice. The results indicated that 55% of the gene was silenced at a CD/siRNA weight ratio of 12, and when the weight ratio was increased to 50–100, the gene knockdown reached 85%. However, at higher ratios, the cell viability decreased. Kim et al. [127] used highly fluorescent PEI/CDs for the delivery of siRNA and for bioimaging (Fig. 6).

For example, dsRNA was tested with three NPs, namely, chitosan, silica and CDs, to target SNF7 and SRC (as mosquito genes)

Table 2
Application of CDs for image-guided gene therapy.

Precursors and surface passivation	Synthesis method	Properties	Zeta potential	Cargo	Cell lines/animal	Major outcomes	Ref
Porphyra polysaccharide – EDA	Hydrothermal	Size: <10 nm, QY: 56.3%	23.54 ± 1.4 mV	pDNA encoding transcription factors Asc11, Brn2 and Sox2	EMSCs	Differentiation of stem cells to neural cells with CDs achieved faster and more efficiently than with all-trans retinoic acid, low cytotoxicity	[113]
PEI and folic acid (FA)	Hydrothermal	Size: 2–9 nm, QY: 42%, uniform dispersion	+23.5 mV	Enhanced green fluorescent protein DNA plasmid (pEGFP)	293 T, HeLa	Low cytotoxicity, bioimaging, targeted gene delivery	[110]
Arginine and glucose	Microwave	Size: 1–7 nm, QY: 12.7%, high solubility, tuneable fluorescence	25.4 ± 0.3 mV	Gene plasmid SOX9	MEFs	Obvious chondogenic differentiation, low cytotoxicity, biocompatibility	[114]
Glycerol and PEI, folate-conjugated reducible PEI	Microwave	Size: 9.0 ± 1.1	4.4 ± 1.7 mV	siRNA (EGFR and cyclin B1)	H460, 3T3, animal	Biocompatibility, sustained gene silencing, stimulus-responsive property	[97]
Citric acid (CA), 1,2-EDA, polycation-b-polyzwitterion copolymer (PDMAEMA-b-PMPDSAH)	Microwave	Size: 2.2 ± 0.3 nm, QY: 41.5%	Depended on polymer/DNA weight ratio: from +10 mv to +35 mV	pDNA	COS-7	High transfection efficiency, bioimaging, high haemocompatibility	[115]
Tetrafluoroterephthalic acid, branched-PEI	Solvothermal	Size: 4.8 ± 0.5 nm	12.6 ± 0.3 mV	pDNA	HEK 293 T, NIH 3T3, COS-7, HepG2, B16F10, A549, Primary 3T3-L1, mESCs	Low cytotoxicity, efficient transfection, enhanced affinity of encapsulated DNA to cytomembrane	[14]
Low molecular weight amphiphilic PEI (Alkyl-PEI2k)	Laser ablation	Size: 10 nm, monodisperse	17.33 ± 1.97 mV	siRNA and pDNA	4T1-luc, 4T1 cells, animal	Low toxicity and good gene transfection effect *in vitro* and *in vivo*	[86]
PEI, 2-((dodecyloxy)methyl) oxirane	Hydrothermal	Size: 3–7 nm	+35.4 ± 1.5 mV	EGFP, siRNA, pDNA, doxorubicin (DOX)	A549	Low cytotoxicity, high transfection efficiency, early cell apoptosis, good drug loading ability	[116]
Glycerol with PEI	Microwave	Size: 5–10 nm, maximum emission: 465 nm	Approximately +30 mV	pDNA	HeLa, PC-3	High cell viability of CD-PEI/Au-PEI carrier, high transfection efficiency (the appropriate size of the complex might facilitate cellular uptake)	[117]
PEI, 2,2,3,3,4,4-hexafluoro-1,5-pentanediol diglycidyl ether	Hydrothermal	Size: 1.5–3.5 nm, QY: 5. 6%	From +30 to 40 mV	Cy5-labelled pDNA	HepG2, HeLa, 7702, A549	High transfection efficiency and cellular uptake, good cell imaging capability under single-wavelength excitation, minimal cytotoxicity	[101]
Glycerol and branched PEI	Microwave	QY: depended on microwave irradiation time	From 0 to +25 mV	pDNA	COS-7, HepG2	Low cytotoxicity, high transfection efficiency	[12]
Citric acid and branched PEI	Microwave	Size: depended on pH	At pH 1, 4 and 8, the zeta potential was +36.5 ± 6.2 mV, +51.8 ± 4.8 mV and +2.7 ± 4.4, respectively.	pDNA and siRNA	A549, A549-Luc, animal	High transfection rate, cell viability was dwindling by increasing concentration of carrier	[118]
Alginate	Hydrothermal	Size: 5–10 nm, QY: 12.7%	+25 mV	Plasmid TGF-β1	3T6	Exhibited strong and stable fluorescence, water-dispersible, high transfection efficiency, negligible toxicity	[119]
Citric acid and tryptophan (Trp)- PEI-adsorbed CD NPs (CDs@PEI)	Microwave	Size: 3.9 ± 0.3 nm, QY: 20.6%	+26.6 ± 1.6 mV	Survivin siRNA	MGC-803	Superior water solubility, excellent biocompatibility, enhanced gene delivery efficiency, induced efficient gene knockdown	[13]
HA and PEI	Microwave	Size and QY: depended on microwave irradiation time	Increase from −5 mV to +44 mV as the weight ratio of CDs/DNA increased	pDNA	HeLa	Low cytotoxicity, high transfection efficiency, strong blue fluorescence under UV light, good intracellular imaging ability	[34]

(*continued on next page*)

Table 2 (*continued*)

Precursors and surface passivation	Synthesis method	Properties	Zeta potential	Cargo	Cell lines/animal	Major outcomes	Ref
Glucose and branched or linear PEI	Hydrothermal	Size: 3.5 ± 0.9 nm, QY of CDs with branched PEI: 2.861%, with linear PEI: 2.439%	–	pDNA	HEK 293T	The branched PEI-modified CDs exhibited higher gene transfection efficiency than linear PEI and naked pDNA	[25]
HA and PEI	Hydrothermal	Size: approximately 2.25 nm, QY: 12.4%	For CDs: approximately +27 mV, for CDs/pDNA: about +16 mV	pDNA	MGC-803, Hela	Biocompatibility, excellent gene condensation capability	[120]
PEG and PEI	Microwave	Size: 3.7 ± 0.7 nm	+15 ± 8 mV	dsRNA of two target genes (SNF7 and SRC)	*Aedes aegypti* larvae	Nontoxicity, gene suppression	[121]

for the control of *Aedes aegypti* larvae. Based on the evaluation of mortality caused by dsRNA targeting of each carrier on three different days, the CDs/dsRNA, showed the most efficient target-gene knockdown among the vectors, with mortality from 38% and 32% for CDs/dsAaSRC and CDs/dsAaSNF7 on the third day to 53% and 75% by the seventh day. For chitosan NPs, after seven days, the mortality for the dsAaSRC and dsAaSNF7 treatments reached 27 and 47%, respectively, and amine-functionalized silica NPs/dsAaSRC caused no mortality or efficacy [121].

For siRNA delivery, the dissociation of the nucleic acid from the carrier is important because the molecules are smaller than plasmid DNA and their association with the carrier might be stronger. In these cases, looser nano complexes have shown higher transfection efficiencies than stronger complexes. Hence, balancing association/dissociation affinity is a key point in the design of an efficient siRNA carrier.

How carbon dots enter cells to deliver nucleic acids

NPs are able to enter cells via different pathways. The possible internalization pathways of NPs include phagocytosis, macropinocytosis, and endocytosis [129]. Receptor-mediated endocytosis (RME) is known to be a major uptake pathway for NPs. RME may involve the participation of clathrin-coated vesicles, caveolae internalization, or other lesser-known mechanisms [130]. Clathrin- and caveolin-dependent endocytosis involves complex biochemical signalling cascades. The size, shape and other physicochemical properties of NPs are correlated with the rate and quantity of NP cellular uptake [131]. The incubation of CDs with different mammalian cell lines showed that small-scale carbon-based materials could readily penetrate the cell membrane and exhibit favourable biocompatibility. The internalization mechanisms of CDs/pDNA nanocomplexes have been investigated by employing four cellular uptake inhibitors: filipin III, glucose, 5-(N,N-dimethyl)-amiloride (DMA), and chlorpromazine hydrochloride (CPZ). The results showed that no fluorescence was emitted by cells when they were cultivated with filipin III, glucose, and CPZ, whereas cells treated with DMA exhibited strong fluorescent intensity [114,132,133].

Accordingly, CDs/pDNA NPs could be internalized via both caveolae- and clathrin-mediated endocytosis and could enter the nuclei to achieve effective gene expression, whereas macropinocytosis plays a minimal role (Fig. 7).

The internalized CDs are located mainly within endo-lysosomal structures and the Golgi apparatus, and a portion of them enter the nucleus; they can also be actively transported to the cell periphery and exocytosed.

Conclusions and future perspectives

It appears that the balance between the positive charge of the carrier and the induced toxicity plays a crucial role in polymer-based nano delivery systems. In other words, a positive charge is necessary for interactions between the nucleic acid material and the vehicle. This process is called vector packaging and occurs outside the cells, leading to the formation of NPs and protection of the nucleic acid against degradation. However, nucleic acid materials must be able to dissociate from the vehicle inside the cells. Therefore, vector unpackaging (dissociation) can be considered a major step in successful gene delivery. CDs developed through several inexpensive, eco-friendly and facile routes have exhibited fine biocompatibility, high quantum yield and stable fluorescence. The positive charge of CDs led to excellent DNA condensation, high transfection efficiency and negligible toxicity. CDs as non-viral gene vectors are shedding light on gene therapy via the delivery

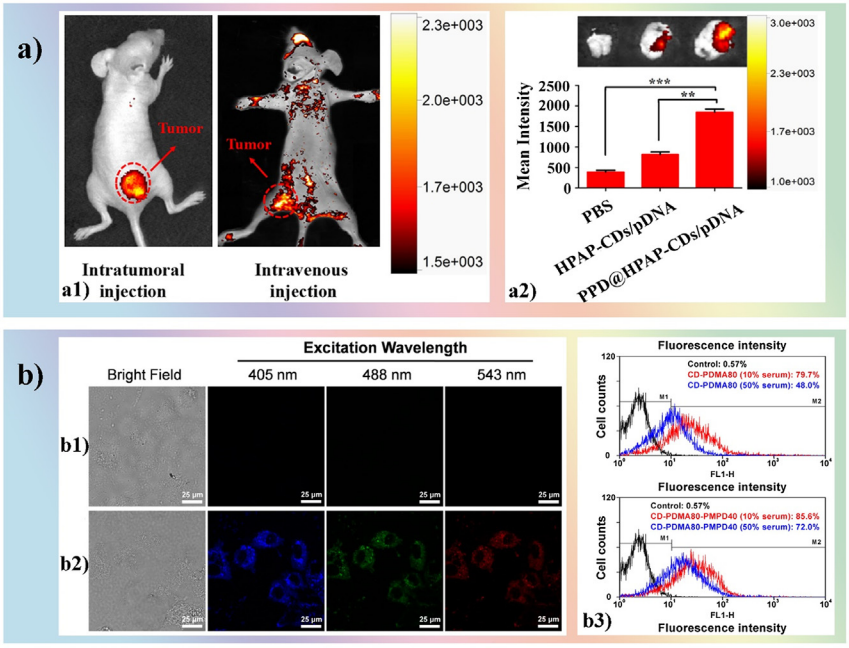

Fig. 5. (a1) Imaging of drug accumulation after PPD@HPAP-CDs/pDNA topical injection and IV administration after 8 h. (a2) quantitative distribution analysis and tumour imaging after treating by IV injection of PBS, HPAP/CDs/pDNA, and PPD/HPAP/CDs/pDNA. Reprinted with permission from [101]. Copyright 2018 American Chemical Society. (b1) negative control (COS-7 cells without transfection) and (b2) samples (COS-7 cells after CD-PDMA80-PMPD40/pDNA transfection). (b3) COS-7 cells enumeration test of cell mixed with CD/PDMA80/pDNA and CD/PDMA80/PMPD40/pDNA samples. Reprinted with permission from [115]. Copyright 2014 American Chemical Society.

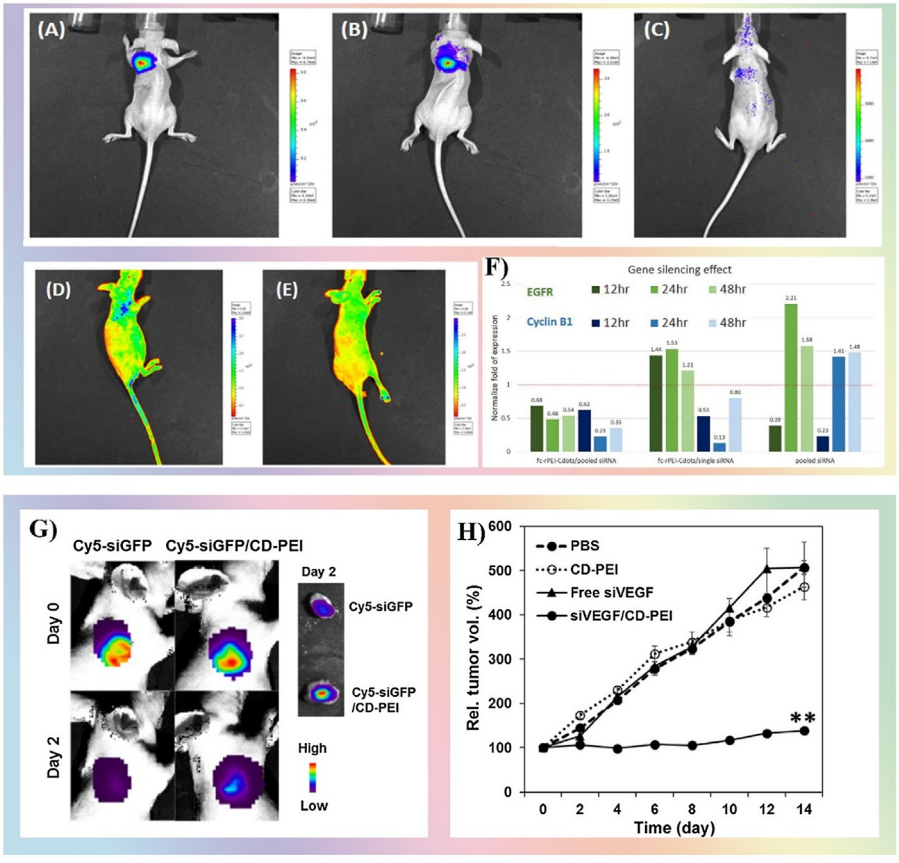

Fig. 6. Bioluminescent imaging of luciferase inhibition after fc/rPEI/CDs delivery in luciferase-expressing H460 lung carcinoma. The image of the lungs at the time of treatment (A), after 7 days (B) and after 10 days (C). Accumulation at lung region of the fc/rPEI/CDs/pooled siRNA after aerosol delivery (D), PBS as a control sample (E). (F) Gene silencing after delivery of fc/rPEI/CDs/pooled siRNA, fc/rPEI/CDs/single siRNA, and pooled siRNA in H460 for 12 h, 24 h, and 48 h. Reprinted by permission from Nature, Scientific Reports [97], Copyright 2016. Bioimaging of tumour treatment by free Cy5-siGFP and the Cy5-siGFP/PEI/CDs (G). The tumour volumes measuring after intravenous administration of PBS, PEI/CDs, free siVEGF, and siVEGF/PEI/CDs (H). Reprinted by permission from Springer, Nano Research [127], Copyright 2017.

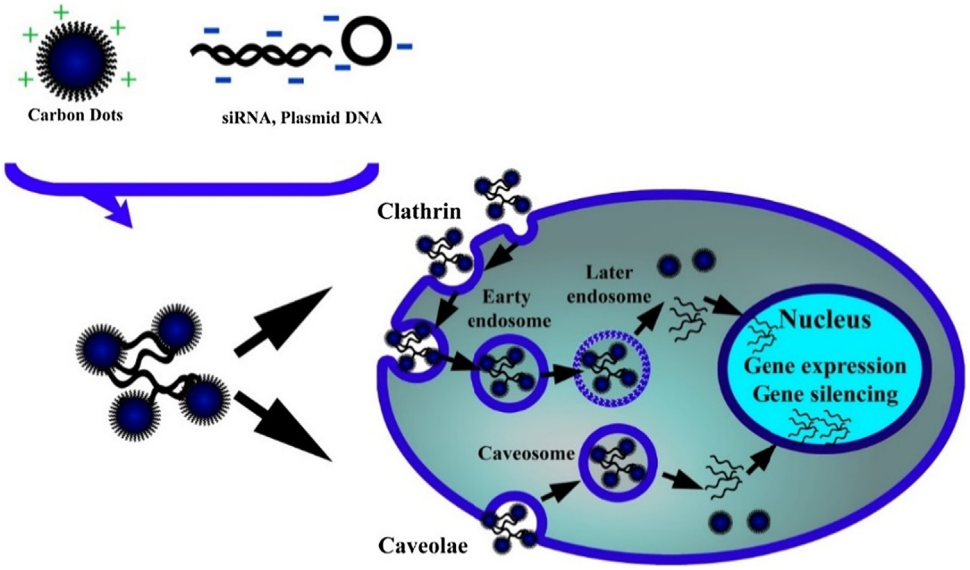

Fig. 7. Internalization mechanisms of CDs/pDNA nanocomplexes.

of plasmids and noncoding RNAs. The PL properties of CDs also permit easy tracking of cellular uptake. Taken together, the evidence shows that cationic CDs hold great potential in theranostics and image-guided gene delivery, due to their dual role as efficient non-viral gene vectors and bioimaging probes. Based on their interesting properties, CDs have great potential as nucleic acid nanocarriers in preclinical and clinical studies that will hopefully result in the bench-to-bedside translation of biocompatible CDs.

Conflict of interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

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Reza Mohammadinejad studied Biotechnology at the Graduate University of Advanced Technology, where he obtained a M.Sc. in 2013. In 2016 he started his PhD in the Pharmaceutics Research Center at the Kerman University of Medical Sciences. Here, his research interests lie in the development of CRISPR gene editing therapies, gene delivery systems and nanobiotechnologies.

Arezoo Dadashzedeh was born in Tabriz, Eastern Azerbaijan, Iran, in December of 1990. She was in Tabriz until the end of high school and pre-university, and then traveled to Tehran to continue her education in university. Eventually, in 2015, she graduated with MSc in biomedical engineering in the tissue engineering field from Amirkabir University of Technology. Her research interests are Drug Delivery systems, Bioprinting and Bioinks, Wound dressing and Biomaterials for Tissue Engineering.

Saeid Moghassemi was born in Borujerd, Lorestan, Iran, in September, 7, 1991. He migrated to the capital city of Iran to pursue his education in his favorite field, biomedical engineering. Finally, in December of 2015, he completes his MSc of biomedical engineering in the biomaterial engineering field from Amirkabir University of Technology. His research interests have focused on Novel Drug Delivery systems, Bioprinting and Biomaterials for Tissue Engineering usages.

Milad Ashrafizadeh was born in Mashhad, Iran in 1994. He is studying veterinary medicine at University of Tabriz. Currently, he is working on stem cells and nutritional supplements against nicotine, lead and cadmium toxicity. Recently, he has engaged in gene therapy and therapeutics delivery.

Ali Dehshahri is currently associate professor of pharmaceutical biotechnology at Shiraz University of Medical Science, Iran. He obtained his PhD at Mashhad University of Medical Sciences, Iran, in 2009, working with Prof. Mohammad Ramezani. In his thesis, he investigated the role of polymer amine content on its efficiency for gene delivery. As a distinguished Ph.D. student, he was awarded a short-term research grant from the Iranian Ministry of Health to pursue his investigation at LMU, Munich, Germany under the supervision of Prof. Ernst Wagner on polymeric nanoparticles for siRNA delivery. In 2014, Dehshahri accepted an Associate Professor position at Shiraz University of Medical Sciences, where he has been since that time.

Abbas Pardakhty received his Pharm.D. from Mashhad University of Medical Sciences and a PhD in Pharmaceutic from Isfahan University of Medical Sciences, Iran, under the supervision of Prof. Jaleh Varshosaz. He passed a sabbatical 6-month course on Gene Delivery at Strathclyde University, UK, with Prof. Ijeoma Uchegbu (2000) and another one with Prof. Gerrit Borchard (2010), Switzerland, at Université de Genève on niosomal vaccine of cutaneous leishmaniasis. He's now a Prof. in Kerman Faculty of Pharmacy and also has 5 years' experience as the Deputy of Research and Technology of Kerman University of Medical Sciences, Iran, from 2014. His current research focuses on the development of lipid vesicular systems for drug and gene delivery.

Hosseinali Sassan studied Genetic engineering and molecular biology at the Universiti Putra Malaysia (UPM) where he obtained a PhD in 2006. He joined the Shahid Bahonar University, where he is currently an Assistant Professor of Molecular biology. His general research interests lie in the genetic engineering.

Seyed-Mojtaba Sohrevardi received his Pharm. D. in 1997 and Board Certify Pharmacotherapy Specialist (BCPS) from Tehran University of Medical Sciences (Tehran, Iran) in 2004. After 6 months sabbatical period on the Atherosclerosis prevention in the Robarts Research Institute, University of Western Ontario, Canada under supervision of Prof. J. David Spence, he joined Kerman University of Medical Sciences as a faculty member. After 7 years, He joined at the Shahid Sadoughi University of Medical Sciences (Yazd, Iran). He is an associated Prof. in Pharmacotherapy. His research interests are drugs safety, clinical trials, pharmacokinetic and pharmacogenetics studies. He is working at the Stroke Prevention and Atherosclerosis Research Centre, Robarts Research Institute, University of Western Ontario as a research scholar in 2018–2019.

Ali Mandegary completed his PhD at Tehran University of Medical Sciences with Professor Mohammad Hossein Ghahremani (2008). After 6 months sabbatical period on the leukemic stem cells with Martin Ruthardt at Johann Wolfgang Goethe University, Frankfurt, Germany (2008), he joined the Kerman University of Medical Sciences, where he currently is Associate Professor of Toxicology and Pharmacology. He has focused on anti-cancer drugs, genetic polymorphism, target therapy using nanomaterials, and toxicity evaluation of nanoparticles.