Efficient *in vitro* and *in vivo* pulmonary delivery of nucleic acid by carbon dot-based nanocarriers

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Philippe Pierrat [a, *], Rongrong Wang [a], Dimitri Kereselidze [a], Marie Lux [a], Pascal Didier [b], Antoine Kichler [a], Françoise Pons [a], Luc Lebeau [a, *]

[a] Laboratoire de Conception et Application de Molécules Bioactives, CNRS – Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, BP 60024, 67401 Illkirch, France
[b] Laboratoire de Biophotonique et Pharmacologie, CNRS – Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, BP 60024, 67401 Illkirch, France

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ABSTRACT

Cationic carbon dots were fabricated by pyrolysis of citric acid and bPEI25k under microwave radiation. Various nanoparticles were produced in a 20–30% yield through straightforward modifications of the reaction parameters (stoichiometry of the reactants and energy supply regime). Particular attention was paid to the purification of the reaction products to ensure satisfactory elimination of the residual starting polyamine. Intrinsic properties of the particles (size, surface charge, photoluminescence and quantum yield) were measured and their ability to form stable complexes with nucleic acid was determined. Their potential to deliver plasmid DNA or small interfering RNA to various cell lines was investigated and compared to that of bPEI25k. The pDNA *in vitro* transfection efficiency of these carbon dots was similar to that of the parent PEI, as was their cytotoxicity. The higher cytotoxicity of bPEI25k/siRNA complexes when compared to that of the CD/siRNA complexes however had marked consequences on the gene silencing efficiency of the two carriers. These results are not fully consistent with those in some earlier reports on similar nanoparticles, revealing that toxicity of the carbon dots strongly depends on their protocol of fabrication. Finally, these carriers were evaluated for *in vivo* gene delivery through the non-invasive pulmonary route in mice. High transgene expression was obtained in the lung that was similar to that obtained with the golden standard formulation GL67A, but was associated with significantly lower toxicity. Post-functionalization of these carbon dots with PEG or targeting moieties should significantly broaden their scope and practical implications in improving their *in vivo* transfection efficiency and biocompatibility.

1. Introduction

Gene therapy is regarded as a promising treatment for many diseases, whether acquired (*e.g.* AIDS or cancer) or inherited through genetic disorder (cystic fibrosis, Parkinson's disease…). The basic concept underlying gene therapy is that disease may be treated by the transfer of genetic material into specific cells of a patient in order to enhance gene expression (DNA transfection) or to inhibit the production of a target protein by using the RNA interference machinery (*i.e.* siRNA transfection). Due to the size and the polyanionic nature of nucleic acid, its fragility towards nucleases, and the negative potential of the cell membrane surface, gene therapy requires the use of a proper carrier for safe and efficient intracellular delivery of the therapeutic payload. Though viral vectors offer greater efficiency of gene delivery, fundamental problems are associated with these carriers and many clinical trials in which they were used have been interrupted because of unexpected adverse effects [1–4]. Furthermore, severe limitations are encountered with respect to production and scale-up procedures. This has encouraged investigators to develop other potential scaffolds for introducing exogenous nucleic acids into targeted tissues. These so-called nonviral (or synthetic) carriers are typically based on cationic lipids or polymers, dendrimers, polypeptides, or solid nanoparticles, which can complex with

* Corresponding authors. Laboratoire de Conception et Application de Molécules Bioactives – UMR 7199, CNRS – Université de Strasbourg, Faculté de Pharmacie, 74, route du Rhin, CS 60024, 67401 Illkirch cedex, France. Tel.: +33 03 68 85 43 03; fax: +33 03 68 85 43 06.

E-mail address: llebeau@unistra.fr (L. Lebeau).

negatively charged nucleic acid to form nano-sized particles [5]. Nonviral carriers have several advantages such as ease of synthesis, cell/tissue targeting, low immunogenicity and capacity to transport plasmid of unrestricted size. The most severe bottleneck in the clinical use of synthetic vectors is their low *in vivo* transfection efficiency and none of these vectors has received FDA or EMA approval to date. Therefore, efforts still are required for developing carriers allowing improved cell uptake, optimized endosome escape and migration of nucleic acid through the cytoplasm (to the cell nucleus or to the cytosolic RNA-induced silencing complex, RISC), together with dissociation of nucleic acid from the carrier for proper expression.

The recent discovery of carbon dots (CD) and the rapid advances in their synthetic preparation do offer a unique opportunity for investigating their potential as a new family of especially exciting carriers for nucleic acid delivery. These carbonaceous quantum dots combine several favorable attributes of traditional semiconductor-based quantum dots (namely, nanoscale size, size- and wavelength-dependent luminescence emission, resistance to photobleaching, ease of bioconjugation) without incurring the burden of intrinsic toxicity or elemental scarcity, and without the need for stringent, costly, or inefficient preparation steps [6]. Furthermore, CD surface passivation by amino compounds as required for obtaining exacerbated intrinsic fluorescence properties [7] allows straightforward installation of cationic charge density at the surface of the nanoparticles. This was investigated by Liu et al. who recently introduced polyethyleneimine (branched PEI 25 kDa, bPEI25k), a golden standard transfection reagent (for recent reviews, see Refs. [8,9]), for CD passivation [10]. The CD were prepared from bPEI25k and glycerol under microwave radiation. The resulting water-soluble brightly fluorescent nanoparticles exhibited *in vitro* DNA transfection efficiency comparable to control bPEI25k, with lower toxicity. These results are the very first to suggest the potential of CD for applications in gene delivery. Since then, only three other reports have described the use of engineered carbon dots for transfection purpose. In 2013, Kim et al. reported on a ternary complex between PEI-functionalized CD, PEI-functionalized gold nanoparticles and plasmid DNA (pDNA) for *in vitro* transfection and real-time monitoring of plasmid cellular trafficking [11]. In this study, the PEI-functionalized CD were prepared according to the procedure previously reported by Liu. More recently, Wu et al. described the hydrothermal preparation of CD by employing bPEI as carbon source [12]. The resulting cationic nanoparticles proved efficient for *in vitro* transfection of MCF-7 cells using EGFP as a reporter gene. Lastly, Chen et al. described the post-functionalization of CD with Alkyl-PEI2k (bPEI2k with 11% of nitrogen atoms bearing a dodecyl substituent) and their use for *in vivo* delivery of DNA or siRNA [13]. Thus they demonstrated gene expression and silencing, respectively after intratumoral administration of CD–pDNA and CD–siRNA complexes in a xenografted tumor mouse model. Though the model used is a highly specific one, it provided the first proof of concept that CD can mediate transfection *in vivo*.

Herein, by employing citric acid and bPEI25k as carbon source, we established a simple and passivation-free route to cationic CD. The experimental conditions for the one-step pyrolysis of the carbon source under microwave radiation were systematically investigated. The as-produced carbon dots were purified by dialysis under specific conditions and did not require any post-functionalization for efficient DNA or siRNA *in vitro* transfection. The potential of these nanoparticles for *in vivo* DNA transfection by the non-invasive airway administration route was investigated.

2. Materials and methods

2.1. Materials

Citric acid was from Merck (Darmstadt, Germany). Branched PEI (MW 25 kDa, bPEI25k) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were from Sigma–Aldrich (St Louis, MO, USA). Cholesterol (3-aminopropyl) [4-{(3-aminopropyl)amino}butyl] carbamate (GL67) was synthesized according to a described procedure [14]. DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) and DMPE-PEG$_{5000}$ (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethyleneglycol)$_{5000}$]) were from Avanti Polar Lipids (Alabaster, Al, USA). To generate GL67A, the three lipids were formulated at 1:2:0.05 (GL67:DOPE:DMPE-PEG$_{5000}$) molar ratios [15]. Dialysis membranes were from Spectrum laboratories (Rancho Dominguez, CA, USA). A549 cells (human lung carcinoma; CCL-185), and NIH/3T3 cells (mouse embryo fibroblast; CRL-1658) were obtained from ATCC-LGC (Molsheim, France). pCMV-FLuc expression plasmid (5.5 kbp, BD Biosciences Clontech, Franklin Lakes, NJ, USA) was used as cytosolic luciferase reporter gene to monitor DNA transfection activity *in vitro* [16]. This plasmid encodes the *Firefly (Photinus pyralis) luciferase* gene under the control of a strong CMV promoter. To monitor DNA transfection *in vivo*, pCMV-GLuc (5.7 kbp, Nanolight Technologies, Pinetop, Az, USA) was used as luciferase reporter gene. This plasmid encodes the *Gaussia princeps luciferase* gene [17]. Cy5-labeled DNA (DNA-Cy5) was prepared from pCMV-GLuc and Cy5-*Label*IT reagent from Mirus Corp. (Madison, WI), following the manufacturer's instructions. The A549 cell line was transformed to stably express the *Photinus pyralis* luciferase gene originating from the pGL3 plasmid (Clontech, Mountain View, CA) to assess siRNA delivery [18,19]. The pGL3 plasmid encoded as well for a gene conferring resistance to the antibiotic G418. This antibiotic was thus used to select the transfected A549-Luc cells. Luciferase gene silencing experiments were performed with an RNA duplex (siLuc) of the sense sequence: 5′-CUU ACG CUG AGU ACU UCG A. Control untargeted RNA duplex (sic) was of sense sequence: 5′-CGU ACG CGG AAU ACU UCG A. Both RNAs as well as Cy5-labeled siLuc (siRNA-Cy5) were from Eurogentec (Angers, France). DNA concentration refers to phosphate content whereas siRNA concentration refers to duplex content. Fetal bovine serum (FBS), culture media (Dulbecco's Modified Eagle Medium, DMEM), Hank's Balanced Salt solution (HBSS), and supplements were from GIBCO-BRL (Cergy-Pontoise, France). Lysis and luciferin solutions for monitoring luciferase activity were purchased from Promega (Charbonnières, France). Coelenterazine was from Alfa Aesar (Bisheim, France). Eight-week-old male BALB/c mice were purchased from Charles River Laboratories (Saint-Germain-sur-l'Arbresle, France). They were housed in polycarbonate exhaust ventilated cages (M.I.C.E.® cages, Animal Care Systems) at a rate of 4 mice per cage, with bedding made from spruce wood chips (Safe, Villemoisson, France). Ventilation in the cages was set to 10–12 changes per hour, according to the manufacturer's recommendations. The animal room was maintained under controlled environmental conditions, with a temperature of 20 ± 2 °C, a relative humidity of 50 ± 10% and a 12 h/12 h light/dark cycle (lighting 07:00–19:00). Food (standard diet 4RF21, Mucedola, Milan, Italy) and tap water were available *ad libitum*. The animals were acclimatized for 1 week before the initiation of the study. Animal experiments were performed in accordance with the European Union guidelines for use of laboratory animals and with the approval of the government body that regulates animal research in France (agreement number: AL/23/30/02/13).

2.2. Methods

2.2.1. Preparation of the CDs

The carbon dots were synthesized by microwave-assisted pyrolysis of citric acid. Briefly and unless otherwise stated, citric acid (0.50 g) was mixed with bPEI25k (0.25 g) in HCl 0.1 N (5 mL) in a 100 mL erlenmeyer flask. The homogeneous solution was submitted to microwave radiation in a 23 L domestic oven with a triple wave distribution system (TDS Silver ME82V, Samsung). Power output and reaction time were selected manually (typically, 700 W for 150 s). The reaction mixture was then cooled to room temperature, ultrapure water (5 mL) was added, and the resulting solution was centrifugated (5000 rpm, 5 min). The supernatant was loaded into a dialysis device (MWCO 3500 Da) and equilibrated against 500 mL HCl 0.1 N for 24 h (dialysate was replaced at 2, 6, and 12 h), and against ultrapure water for 48 h (dialysate was replaced once every 12 h). The residue was lyophilized to yield powdered material. Fourier transform infrared spectroscopy (FT-IR) was performed on a FT-IR Nicolet 380 spectrometer. NMR spectra were recorded on a Bruker 400 MHz Avance III instrument. 1H and 13C NMR chemical shifts δ are reported in ppm relative to internal reference (^{1}H: HDO at 4.78 ppm; ^{13}C in D$_2$O: (CH$_3$)$_3$COH at 30.83 ppm). The composition of the CDs was determined by elemental analysis on an Elementar Vario EL III apparatus.

2.2.2. Preparation of the CD-nucleic acid complexes

Procedure A: The DNA complexes formulated at various weight ratios for *in vitro* evaluations were prepared by mixing equal volumes of stock solutions of CD and pDNA (prepared at the adequate concentration in ultrapure water). The complexes were allowed to form for 30 min at room temperature without handling. Finally, the mixture was homogenized by pipetting up and down and subsequently used for