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Dinuclear metal(II) complexes of polybenzimidazole ligands as carriers for DNA delivery

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ABSTRACT

A metal-based nonviral carrier for DNA-transfer in gene therapy was synthesized and characterized. The strong intermolecular π–π contacts are common in the observed dinuclear Cu^{2+} and Co^{2+} complexes of polybenzimidazole ligands. The affinity assays indicated that the strong binding of the complexes to DNA is driven by both electrostatic attractions between the complexes and DNA and the intercalation of the ligands between DNA base pairs. The typical *in vitro* studies showed that micromolar doses of each complex tested can efficiently and rapidly condense free DNA, either in linear or circular state, from solutions into well defined and globular nanoparticles with varied sizes. Therefore, the formation and dissociation of the DNA condensates were explored in detail under different conditions, indicating that the sizes of DNA condensates can be regulated by changing both doses and incubation time for the different dinuclear complexes. Since the intermolecular π–π interactions in the DNA-bound complexes may be a key force to drive DNA condensation, we propose a new DNA condensation model that is essentially distinct from that proposed for the most studied multivalent cationic reagents used in DNA condensation. The cellular uptake experiments successfully performed with the DNA condensates including plasmid pGL3 control vector that strongly expresses luciferase in many types of mammalian cells, as well as cytotoxicity evaluation, demonstrated the potentiality of the dinuclear metal(II) complexes as a new nonviral gene carrier. Finally, the dinuclear Co^{2+} complexes of polybenzimidazole ligands are suggested to be the most potential nonviral gene carriers that are not impacted by serum proteins.

1. Introduction

Gene therapy may be described as the delivery of nucleic acids by means of a vector to patients for some therapeutic purpose. Its potential to benefit human health is tremendous because almost all human diseases have a genetic component from untractable genetic disorders to cancer and heart diseases. Unfortunately, clinical trials have failed to deliver [1]. Developing effective and safe carriers for gene delivery remains a major challenge so far. Gene carriers condense or pack and protect DNA and oligonucleotides, because DNA and oligonucleotides are rapidly degraded by serum nucleases in the blood when injected intravenously [2]. There are two kinds of carriers for gene delivery: viral and nonviral. Viral carriers exhibited high efficiency at delivering both DNA and RNA to numerous cell lines [3]. However, limitations with viral approaches include small cargo capacity, resistance to repeated infection, difficulty in production and quality control, and low safety [4]. For these reasons, research attention has been attracted to nonviral approaches that make use of synthetic chemical carriers have the potential to overcome many of inherent limitations or challenges of viral carriers [5–7].

Nonviral carriers to enable improved DNA stability and cellular uptake of DNA condensates, including multivalent cations and organic polymers such as polyamine, polysaccharides, and cationic lipids, peptides, and dendrimers, all offer potential routes for condensing DNA through electrostatic interactions with DNA [5–10]. Some of the organic nonviral carriers have been introduced into clinical trials [6]. Cationic metal complexes are also a kind of potential nonviral gene carriers, and do not yet arouse full interest from investigators [11–16]. Such nonviral carriers were reported in detail only for $[Co(NH_3)_6]^{3+}$ (hexammine cobalt(III) cation), a mononuclear metal complex that is nonreactive to DNA [11,17–26]. The complex condenses the plasmid pUC12, calf thymus DNA, λDNA, and polynucleotides into nanoparticles of 39–45 nm under neutral conditions, as indicated by dynamic light scattering

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(DLS) [17–26]. Although hexammine cobalt(III) and spermidine bear the same number of three positive charges under neutral conditions, the former condenses DNA 5-fold more efficiently than spermidine, with the size of the condensates generally smaller than that formed with spermidine [17], indicating that structural features and charge density of the condensers have remarkable effects on the DNA condensation and size of the nanoparticles formed. The $[Co(NH_3)_6]^{3+}$-induced DNA condensates that contain one to multiple DNA molecules were observed to exhibit frequently well-defined and classical toroidal, and infrequently rod-like and spheroidal profiles in morphology under transmission electron microscope (TEM) [23,27]. Therefore, to understand the mechanism of $[Co(NH_3)_6]^{3+}$-induced DNA condensation, the fine structure of DNA toroids was examined with cryoelectron microscopy [28]. A time study indicated that the transformation can occur between the toroidal and rod-like DNA condensates [29]. The static loops within the condensed DNA molecules were shown to act as a nucleation site for DNA condensation and are responsible for defining the size and morphology of the DNA condensates. Moreover, the reaction conditions including ionic strength and solvent effects were observed to be capable of exerting significant influence on the DNA compaction and its kinetics [21,26,27,30,31]. On the other hand, the thermodynamic studies performed with isothermal titration calorimetry showed that the DNA condensation follows an electrostatic mechanism [32]. However, the experiments of cellular internalization are not reported for the nonviral metal-based carriers for DNA delivery so far.

However, the most fundamental barriers currently associated with synthetic gene carriers, which need to be overcome prior to clinical trials, include the lack of reproducible and scalable formulation, low stability in biological fluids, proneness to aggregation, long term storage and properly reproducible transfection, and DNA size-dependent delivery [1]. Nonviral gene carriers have the potential to create viable pharmaceuticals from nucleic acids. Although increasing effort is put into development of nonviral gene carriers, some of fundamental questions on DNA condensation and delivery remain to be solved, and which type of nonviral carrier system would be most appropriate to develop also remains to be explored [1,6]. We made here an attempt at designing new nonviral gene delivery systems that comprise new classes of defined chemical components and might overcome the fundamental barriers by using the dinuclear metal(II) complexes. We have reported that the dinuclear Cu^{2+} complexes of a polybenzimidazole ligand, dtpb (1,1,4,7,7-pentakis(2′-benzimi-dazol-2-ylmethyl)triazaheptane), can dramatically promote DNA condensation from solutions into well defined nanoparticles under neutral conditions [12]. The work presented reports first the interactions of the dinuclear Cu^{2+} and Co^{2+} complexes of two polybenzimidazole ligands, dtpb and egtb (N,N,N′,N′- tetrakis(2′-benzimidazol-2-yl-methyl)-1,4-diethylene amino glycol ether) with DNA, and DNA condensation mechanism. Then, the cellular uptake and cytotoxicity of DNA condensates are evaluated by intracellular expression of luciferase-encoding gene and MTT assay. The results reveal that some of dinuclear metal complexes are a potential nonviral carrier for DNA delivery in gene therapy, although they have been shown to be capable of efficiently cleaving both DNA and proteins via hydrolytic pathways under the tested conditions [33–36].

2. Materials and methods

2.1. Synthesis and characterization

The ligands egtb and dtpb were prepared according to the previously reported methods [37,38]. Their dinuclear complexes $[Cu_2(egtb)(ClO_4)_2(H_2O)_2](ClO_4)_2$ (**1**), $[Cu_2(dtpb)Cl_2(H_2O)](NO_3)_2$ (**2**), and $[Co_2(egtb)Cl_2](ClO_4)_2$ (**4**) were synthesized and characterized according to our reported procedures [12]. The preparation and structural parameters of $[Co_2(dtpb)Cl_3]Cl$ (**3**) are described in detail in the Supporting Information.

2.2. Determination of affinity for DNA

First, the binding equilibrium constants (K_b) of each complex to calf thymus DNA (ctDNA, i.e., dsDNA, double-stranded DNA) and ssDNA (single-stranded DNA) were determined by UV–vis absorption titration. All reactions containing each complex of 8 μM and ctDNA or ssDNA (24 mer) of 0.25–27 μM (in base pairs or bases) were incubated for 1 min at 37 °C in 20 mM Tris-HCl buffer (pH 7.4), and the absorption spectra (200–450 nm) of the resulted solutions were measured on an analytikjena SPECORD 210 spectrophotometer. Then, the fluorescence quenching is used to qualitatively examine the interactions between each complex and DNA. To monitor the influence of DNA on the intrinsic fluorescence of the complexes, each complex of 8 μM was incubated for 1 min at 37 °C with ctDNA of 0.25–27 μM in the buffer (pH 7.4), and the emission spectra (300–500 nm, excited at 271 nm) of the solutions were measured on a JASCO spectrofluorometer. To monitor the influence of each complex on the fluorescence of ethidium bromide (EtBr) bound to DNA, 8 μM ctDNA and 20 mM EtBr were incubated for 1 min at 37 °C with or without each complex of 0.8–8.0 μM and the emission spectra (530–700 nm, excited at 518 nm) of the mixtures were measured on the spectrofluorometer.

2.3. Preparation and examination of DNA condensates

The reactions containing λDNA, ctDNA, pBR 322 DNA or ssDNA (24 mer) of given amounts and each dinuclear metal(II) complex of varied doses were incubated for variable periods at 37 °C under neutral conditions (pH 7.4) prior to measurements. The resulted DNA condensates were immediately examined by light scattering to evaluate their sizes and observed under TEM for visualization. Examination of DNA condensation was carried out for each sample and each control with electrophoresis mobility shift assays (EMSA) (see Supporting Information), light scattering measurements, and TEM imaging [12]. To monitor the DNA condensation, RALS (right angle light scattering) measurements were made on the spectrofluorometer. Excitation and emission wavelengths all were set at 400 nm (different bandwidths used here). Each spectrum represents an average of 120 accumulations. To evaluate average hydrodynamic diameters of DNA condensates, DLS data were collected with a HORIBA LB-550 dynamic light scattering particle size analyzer. Consecutive measurements (200 times) were made with a cell of 2 mL. The morphology of DNA condensates was examined with TEM. Aliquots of the ongoing DNA condensation reaction mixtures were taken at different time points of incubation or concentration points of reactants for TEM visualization. Samples were placed 5 μL each time on a freshly glow-discharged carbon-coated grid, absorbed for 2 min, and then washed the grid with deionized water before imaging. Grids were directly imaged on a Tecnai G2 20 transimission electron microscope operating at 175 kV and 25–11,00,000× magnification.

2.4. Cellular uptake study

To conveniently observe cellular uptake, the DNA condensates produced by incubating λDNA and each complex of given doses for 30 min were re-incubated for 30 min at 37 °C with the fluorescent dye Greenview (Biorule), and the Greenview-labeled samples were prepared for cellular uptake studies. The DNA-bound form of Greenview via electrostatic interaction emits yellow fluorescence when excited at 365 nm. The human heptocellular carcinoma cell line Hep G2 was suspended in RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The cells were seeded at about 4 000–40 000 cells/well in 24-well culture plates, and maintained at 37 °C in a 5% CO_2 humidified air atmosphere until growth reached 50% confluence as a monolayer. Both freshly prepared and labeled DNA condensates and the controls containing either λDNA or each complex were carefully added to each well, respectively, at a final DNA concentration of 10 μM and final complex concentrations of 0.2, 0.8, 2, 4, and 10 μM in FBS-containing or -free medium, and the cells were maintained at 37 °C in a 5% CO_2 humidified air atmosphere for 24–48 h. The cells were washed three times with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM NaH_2PO_4) after removing the culture medium in the wells. The nucleus-specific green fluorescence dye DAPI (4′,6-diamidino-2-phenylindole, excitement at 470 nm, Promega) was added to each well, and the cells were first incubated for 4 h at 37 °C in a 5% CO_2 humidified air atmosphere, and then washed three times with PBS. The cells were visualized not only by fluorescent microscopy using a Leica DMI3000B inverted microscope equipped with epifluorescent illumination using appropriate filter sets, but also by confocal laser scanning microscopy [39,40]. Fluorescent imaging was taken consecutively using the same image acquisition parameters. Confocal imaging was acquired using a Leica TCS SP5 confocal microscope. All confocal images of DAPI stained nuclei and Greenview stained DNA condensates were collected using 360 and 514 nm excitation light from a He/Ne2 and 488 laser, respectively [41].