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The effect of a nuclear localization sequence on transfection efficacy of genes delivered by cobalt(II)–polybenzimidazole complexes

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ABSTRACT

We have demonstrated that the metal complexes of polybenzimidazoles are emerging likely as a new type of gene-delivery systems based on their strong DNA-condensing ability. However, the *in vitro* transfection efficacy of the DNA condensates formed with the metal complexes was relatively low. The positively charged peptides, such as cell-penetrating peptides and nuclear localization sequences (NLSs), have been reported to be capable of enhancing expression of the transgenes, likely as they promote entrance of their electrostatic complexes with DNA into the nuclear through nuclear pores. Here, we explored expression of the genes transferred by a series of Co(II) complexes in the presence of NLS (PKKKRKV) in normal and cancer cell lines. The results showed that the Co(II) complexes lead to the more pronounced DNA condensation in the presence of NLS than that in the absence of NLS. The binding of NLS prior to addition of the Co complexes can significantly reduce both the size and the population of the condensates at the given Co complexes/DNA ratios, compared with the NLS-free condensates. Meanwhile, the binding of NLS can considerably increase surface positive charges on the DNA nanoparticles. The suitable sizes and high surface positive charges facilitate the entrance of the nanoparticles into cells. Luciferase activity assay indicated that the transfection efficacy of the NLS-bound condensates was five-fold of that of the NLS-free ones in different cell lines, and comparable to that of the condensate formed with the commercially available carrier PEI. Moreover, cell viability assay of the NLS-bound condensates showed lower cytotoxicity than the NLS-free ones. Thus, the combination of NLS and cationic metal complexes might offer a new type of ternary delivery systems.

1. Introduction

The development of nucleic acid-based therapeutics has garnered tremendous interest in the past two decades as a new pharmaceutical agent. The ability to deliver nucleic acids offers the potential to develop therapeutics to cure many diseases, which may be difficult to treat effectively with traditional therapies, e.g., hereditary diseases and cancer. The nonviral carriers (synthetic delivery systems) that can improve both stability and cellular uptake of nucleic acids, including polycations (such as polyamine, polysaccharides, polypeptides), cationic lipids, cyclodextrin, and dendrimers, offer a route for the delivery of nucleic acids [1–10]. The polycation-induced DNA condensation usually provides the positively charged particles that can enter into cells following binding to negatively charged proteoglycans on the outer face of cells [11]. However, the site of action of nucleic acids requires that these molecules are delivered to the interior of the target cells, which greatly complicates delivery strategies and compromises transfection efficiency. In addition, the other fundamental barriers currently associated with the nonviral carriers include the lack of reproducible and scalable formulation, low stability in biological fluids, proneness to aggregation, and nucleic acid size-dependent delivery. Although both the inherent barriers and the poor transfection efficacy of nonviral carriers have forced the majority of clinical trials to employ viral carriers [12], the viral carriers associate, in general, with the significant safety concerns including immunogenicity and insertional mutagenesis [13]. Therefore, it remains to be explored which type of nonviral carriers is most appropriate to develop [1–10,14–31].

Cationic metal complexes are under evaluation as a kind of promising nonviral nucleic acid carriers [32–36]. The early studies indicated that hexammine cobalt(III) cation ($Co(NH_3)_6^{3+}$), a complex that was nonreactive to DNA, effectively induced DNA condensation [32,37–45]. The key factors that control the condensation and morphology of $Co(NH_3)_6^{3+}$-induced DNA condensates have been investigated in details. The metal complex condenses the plasmid

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pUC12, calf thymus DNA, λDNA, and polynucleotides into nanoparticles of 39–45 nm under neutral conditions. The critical concentration of DNA condensation for $Co(NH_3)_6^{3+}$ was 10 μM, and only the amorphous particles were observed when the concentration was ≥500 μM [39]. Moreover, $Co(NH_3)_6^{3+}$ has been observed to condense DNA 5-fold more efficiently than spermidine with the size of the condensates being generally smaller than those formed with spermidine, although both all bears three positive charges under neutral conditions. The DNA condensates that contain one to multiple DNA molecules exhibit frequently well-defined and classical toroidal, and infrequently rod-like and spheroidal profiles under transmission electron microscope (TEM) [42]. The condensation is controlled by both kinetic and thermodynamic processes of $[Co(NH_3)_6]^{3+}$–DNA interactions [41,43]. The thermodynamic study performed by isothermal titration calorimetry (ITC) demonstrated that the condensation followed an electrostatic mechanism [40]. Time courses examined with electron cryomicroscopy indicated that the morphological transformation can occur between the toroidal and rod-like DNA profiles [42,46]. In addition, the cationic Ni(II), Rh(II), and Co(II) complexes, which were observed to be capable of effectively condensing DNA, all contain aromatic heterocyclic ligands as their main ligands because the aromatic ligands can intercalate into DNA base pairs [33,34,47–49]. In fact, an early study has shown that the intercalating aromatic cations can trigger DNA condensation [50].

We have recently reported that the Co(II) and Cu(II) complex series of polybenzimidazoles (pbzims) can efficiently condense DNA and transfer the DNA into cells, as (1) these metal complexes carry permanent positive charges at pH 7.4, and exhibit the high-affinity binding to nucleic acids with multiple interactions being established between the binding partners; (2) the resulting DNA condensates carry positive charges that enhance their association with negatively charged cellular membranes [48,49,51,52]. Moreover, we have demonstrated that the positive charges and benzimidazolyl (bzim) groups on the metal complexes are two key factors to determine their potential as a new type of gene carriers. Indeed, the metal complex-based carriers are emerging likely as a new type of gene-delivery systems prone to systematic structural alteration and chemical tailoring, thereby facilitating their elucidation of structure–activity relationships. However, the results obtained so far indicated that this type of gene carriers is subject to poor transfection efficacy, despite low cytotoxicity [49,51]. Thus, we explored here expression of the genes transferred by a series of Co(II) complexes with variable number of both positive charges and bzim groups in different cell lines, when the nuclear localization sequence (NLS) PKKKRKV (from simian SV40 large tumor antigen) was added into the DNA condensation reactions. The NLSs that carry multiple positive charges have been reported to be capable of enhancing the transfection efficacy of the DNA of interest as they facilitate entrance of their electrostatic complexes with DNA into the nuclear through nuclear pore complexes [53].

2. Materials and methods

2.1. Materials

Ethidium bromide (EtBr), 4,6-diamino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Lipofect (Lipofectamine™ 2000) and PEI (polyethyleneimine) were purchased from Sigma. The NLS peptide (PKKKRKV) was from GL. The plasmid pEGFP-N3 and pGL3 control vectors, and dual luciferase reporter gene assay kit were from Promega. Unless otherwise stated, DNA concentration was expressed in base pairs. The FITC-DNA (fluorescein isothiocyanate-labeled dsDNA oligomer, 5′-GGTCGGAGTCAAC GGATTTGGTCG-3′) were from Invitrogen. All samples were prepared using distilled water that had been passed through a Millipore-Q ultrapurification system. The cell lines HeLa, HT 29, PANC 1, COS 7, NIH 3T3, and HEK 293 were from Boster Biological Technology, LTD.

2.2. Preparation of Co(II)–pbzim complexes

The Co(II)–pbzim complexes (Fig. 1) used here, $[Co(idb)_2]^{2+}$ (**1**), $[Co(ntb)H_2O]^{2+}$ (**2**), $[Co(edtb)]^{2+}$ (**3**), $[Co_2(dtpb)Cl_3]^{+}$ (**4**), and $[Co_2(dtpb)(H_2O)_2]^{4+}$ (**5**), were prepared and characterized according to the reported methods [52]. Here, idb (bis(benzimidazol-2-ylmethyl)amine), ntb (tris(benzimidazol-2- ylmethyl)amine), edtb (N,N,N′,N′-tetrakis(benzimidazol-2-ylmethyl)-1,2-ethanediamine), and dtpb (1,1,4,7,7-pentakis(1H-benzimidazol-2-ylmethyl)-1,4,7-triazaheptane) contain two, three, four and five bzim groups, respectively.

2.3. Electrophoresis mobility shift assay (EMSA)

Binding of the Co(II) complexes to the plasmids in the presence of NLS was determined using agarose gel electrophoresis. 30 μM pEGFP or pGL3 was first mixed with 300 μM NLS for 3 min at 37 °C in 20 mM Tris–HCl buffer (pH 7.4). Then, 0–120 μM of the complexes was added into the resulting mixtures and incubated for 30 min at 37 °C. The NLS-free systems were utilized as controls. Electrophoresis of the resulting DNA assemblies was carried out using 1% agarose gel with 20 μg EtBr in TAE (40 mM Tris–acetate and 1 mM EDTA, pH 8.2) running buffer.

2.4. Right angle light scattering (RALS) assay

To compare effects of the addition of NLS on the Co(II) complex-induced DNA condensation with RALS assay, three groups of samples were prepared in 20 mM Tris–HCl buffer (pH 7.4): (**1**) 5 μM λDNA was first mixed with NLS of varied concentrations, and then the complexes of varied concentrations were added into the resulting solution; (**2**) 50 μM NLS was added into the 5 μM λDNA solution containing the 10 μM Co complexes; (**3**) the mixtures of 50 μM NLS and the 10 μM Co complexes were added into the 5 μM λDNA solution. The 5 μM λDNA solutions containing either NLS or Co complexes were utilized as controls. The λDNA condensation was performed for 10 min or variable periods at 37 °C. The RALS assay was performed on a Cary Eclipse spectrofluorometer. Excitation and emission wavelengths were set all at 350 nm (band pass 2.5 nm) according to diameter distributions of the condensates. Analysis of RALS data was carried out using software ORIGIN.

2.5. Size and ζ-potential measurement

To observe effects of the advance addition of NLS on both size populations and ζ-potentials of the Co(II) complex-induced DNA condensates, 1 μM λDNA was first mixed with 10 μM NLS in 20 mM Tris–HCl buffer (pH 7.4), and then the complexes (1, 2, 4 μM) were added into the mixtures. The 1 μM λDNA solutions containing either NLS or complexes were utilized as controls. The λDNA condensation was performed for 10 min at 37 °C. Both apparent hydrodynamic diameters and ζ-potentials of the DNA condensates were measured by using a ZEN3600 Zetasizer (Malvern, Worcestershire, UK) at 37 °C. Consecutive measurements (100 times) were made for sizes and overall charges of the nanoparticles. To get as accurately an assessment of hydrodynamic sizes as possibly in all cases, samples were measured at a sufficiently low concentration to ensure the absence of interparticulate interactions. Analysis of data was carried out using software ORIGIN.

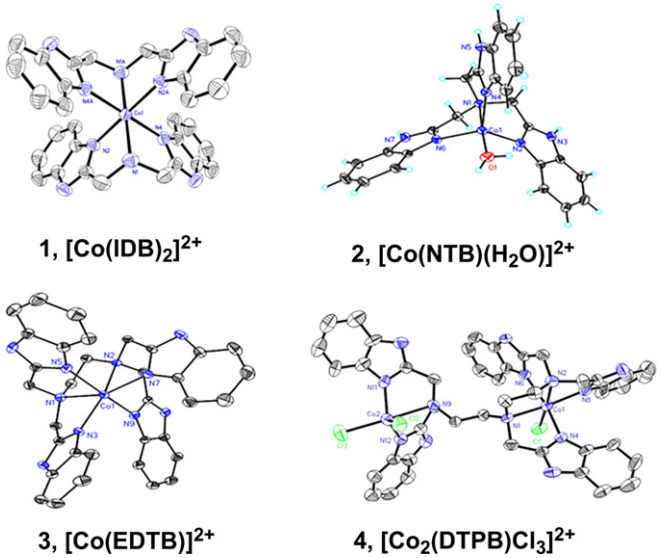

Fig. 1. ORTEP view of the Co(II) complexes **1**–**4**. For clarity, solvent molecules, hydrogen atoms, and counteranions are omitted.