Reversible DNA Condensation Induced by a Tetranuclear Nickel(II) Complex

Xindian Dong,^[a] Xiaoyong Wang,^{*[b]} Yafeng He,^[a] Zhen Yu,^[a] Miaoxin Lin,^[a] Changli Zhang,^[a] Jing Wang,^[b] Yajie Song,^[b] Yangmiao Zhang,^[a] Zhipeng Liu,^[a] Yizhi Li,^[a] and Zijian Guo^{*[a]}

Abstract: DNA condensing agents play a critical role in gene therapy. A tetranuclear nickel(II) complex, $[Ni^{II}_{4}-(L-2H)(H_2O)_6(CH_3CH_2OH)_2]\cdot 6NO_3$ $(L=3,3',5,5'-tetrakis{[(2-hydroxyethyl) (pyridin-2-ylmethyl)amino]methyl}bi$ phenyl-4,4'-diol), has been synthesizedas a nonviral vector to induce DNAcondensation. X-ray crystallographicdata indicate that the complex crystallizes in the monoclinic system with $space group <math>P2_1/n$, a=10.291(9), b=24.15(2), c=13.896(11) Å, and $\beta=$

Introduction

The essence of gene therapy is to deliver extrinsic genes into host cells to replace or override the defective genes causative of genetic abnormalities or deficiencies.^[1] DNA condensation is an essential precondition for transporting a therapeutic gene to its target position,^[2] because compact structures protect DNA from nuclease^[3] and facilitate its entry into cells by endocytosis.^[4] Genes can be delivered by viral or nonviral vectors. Viral vectors usually display high

[a]	X. Dong, Y. He, Z. Yu, M. Lin, C. Zhang, Y. Zhang, Z. Liu,					
	Prof. Dr. Y. Li, Prof. Dr. Z. Guo					
	State Key Laboratory of Coordination Chemistry					
	School of Chemistry and Chemical Engineering					
	Nanjing University, Nanjing 210093 (P.R. China)					
	Fax: (+86)25-83224539					
	E-mail: zguo@nju.edu.cn					
[b]	Prof. Dr. X. Wang, J. Wang, Y. Song					
	State Key Laboratory of Pharmaceutical Biotechnology					
	School of Life Sciences, Nanjing University					
	Nanjing 210093 (P.R. China)					

E-mail: boxwxy@nju.edu.cn Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201001457.

98.175(13)°. The DNA condensation induced by the complex has been investigated by means of UV/Vis spectroscopy, fluorescence spectroscopy, circular dichroism spectroscopy, dynamic light scattering, atomic force microscopy, gel electrophoresis assay, and zeta potential analysis. The complex inter-

Keywords: DNA • electrostatic interactions • gene therapy • nickel • nonviral vector

acts strongly with DNA through electrostatic attraction and induces its condensation into globular nanoparticles at low concentration. The release of DNA from its compact state has been achieved using the chelator ethylenediaminetetraacetic acid (EDTA) for the first time. Other essential properties, such as DNA cleavage inactivity and biocompatibility, have also been examined in vitro. In general, the complex satisfies the requirements of a gene vector in all of these respects.

efficiency in transfection and gene expression; however, some disadvantages, such as host immunogenicity and limited scale of production, have limited their application.^[1a] In contrast, nonviral vectors can be selected from a variety of compounds with low cost of production and desirable flexibility of application; moreover, they are relatively non-immunogenic and do not induce significant inflammatory responses.^[5] Such vectors often possess positive charges, which neutralize negative charges on the phosphates of the DNA backbone and/or reorient water dipoles near DNA surfaces; besides, multivalent cations may cause localized bending or distortion of DNA.^[3,6] These interactions decrease repulsions between DNA segments and induce them to compact into a tight globular structure. Therefore, the primary function of a vector is to promote the condensation of DNA.

A wide range of agents, such as cationic lipids, polymers, dendrimers, polypeptides, and nanoparticles, are known to provoke the condensation of DNA.^[5,7] For example, cationic polyamines and graft copolymers can condense DNA through cooperative electrostatic interactions and enable genetic materials to penetrate the plasma cell membrane.^[8] However, cationic polymers and lipids, as well as dendrimers, may induce cytotoxicity and non-biodegradability; in addition, their poor delivery efficiency and low cell specificity

Chem. Eur. J. 2010, 16, 14181-14189

Fax: (+86)25-83224539

© 2010 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

WILEY CONLINE LIBRARY

- 14181

as DNA vectors need to be overcome before clinical trials.^[8a,9] Transition-metal complexes are emerging as a new class of nonviral vectors; some of them, including complexes of cobalt(III),^[10] copper(II),^[11] and ruthenium(II),^[12] have displayed a remarkable ability to induce DNA condensation. Moreover, the inducing impact of metal complexes on DNA condensation seems to be more effective than that of purely organic condensing agents, in that $[Co(NH_3)_6]^{3+}$ is four times more efficient than spermidine, although they have the same charge (3+).^[13] Despite their considerable merit, some unfavorable properties of metal complexes, such as toxicity and DNA cleavage potentiality, have not been fully addressed in the design strategies. On the other hand, overly stable binding of the above condensing agents to DNA may preclude or greatly reduce its interaction with intracellular molecules and thereby decrease the transfection efficiency relative to that with viral vectors.^[14] Indeed, it has been shown that the property of vector unpacking can limit the efficiency of gene delivery and expression.^[15] The transfection efficiency of stimuli-responsive delivery vectors is higher than that of non-stimulus-responsive vectors.^[16] Therefore, conditional disassembly of the compact DNA should be an additional design principle for nonviral vectors.

In consideration of all of the aforementioned factors, in this study we have designed a tetranuclear nickel(II) complex (1) as a novel gene vector. The rationale for this design is that nickel is an essential element for several animal species and many biological processes, such as lipid metabolism and anaerobic metabolism,^[17] which implies that the toxicity of nickel(II) complexes might be tolerable for patients. More importantly, octahedral nickel(II) complexes do not cleave DNA under oxidative conditions,^[18] suggesting that genes carried by such vectors may not be damaged in the delivery. Cations with a valence ≥ 3 are generally required to induce the condensation of DNA in aqueous solution. Therefore, we chose a ligand with multicoordination centers to ligate nickel(II), which may form a polynuclear complex ion with high positive charges beneficial to DNA condensation. As expected, complex 1 is able to condense DNA at concentrations with marginal toxicity, and the condensed DNA can be released from the vector by treatment with ethylenediamine tetraacetic acid (EDTA) in aqueous solution.

Results and Discussion

Design and synthesis: The biphenol-based ligand L was derived from biphenyl-4,4'-diol by sequential reactions with formaldehyde, hydrogen chloride, and 2-pyridylmethyl-2-hydroxyethylamine (Scheme 1). The obtained ligand contains



Scheme 1. Synthetic route to the ligand. DIEA = diisopropylethylamine.

four potential coordination centers for nickel(II), and therefore could form a multinuclear complex with this metal ion. Since the four alcoholic hydroxyl groups in this ligand are not liable to lose protons in a neutral medium, the complex ion is expected to carry high positive charge, which would strengthen the electrostatic interaction between the complex and DNA. Biphenyl-4,4'-diol was chosen as a linker between the bimetallic centers to keep them at an appropriate distance for more effective interactions with the phosphodiester groups in DNA. In addition, the existence of hydroxyl groups and flexible amine chains may enhance the solubility of the complex. Complex **1** was prepared directly by reacting L with Ni(NO₃)₂·6H₂O in ethanol/water at room temperature. The complex is highly water soluble, which facilitates investigations in aqueous solution.

Crystal structure of complex 1: The structure of complex 1



has been characterized by Xray crystallography. A perspective view of the complex is shown in Figure 1. The crystallographic data are listed in Table S1 in the Supporting Information; selected bond lengths and bond angles are given in Table 1.

X-ray diffraction analysis showed that complex **1** crystalli-

www.chemeurj.org

14182



Figure 1. Crystal structure of the cationic core of complex **1**. Counterions and hydrogen atoms are omitted for clarity.

Table 1. Selected bond lengths (Å) and angles (°) for	complex 1	[a	J
----------------------------------	---	-----------------	-------	-----------	----	---

			-
Ni1-O1	2.111(3)	Ni2–N3	2.039(3)
Ni1-O2	2.062(3)	Ni2-N4	2.075(4)
Ni1-O4	2.132(3)	Ni1-Ni2	3.802(3)
Ni1-O5	2.051(3)	Ni1-Ni1A	12.122
Ni1-N1	2.032(4)	Ni1-Ni2A	11.530
Ni1-N2	2.073(4)	N1-Ni1-N2	82.23(14)
Ni2-O2	2.080(3)	O1-Ni1-N2	82.61(12)
Ni2-O3	2.166(3)	N3-Ni2-N4	83.47(15)
Ni2-O6	2.046(3)	O3-Ni2-N4	80.99(13)
Ni2-O7	2.105(3)	Ni1-O2-Ni2	133.27(14)

[a] Equivalent atoms were generated by symmetry transformation: A = 1-x, -y, -z.

zes in the monoclinic crystal system with space group $P2_1/n$. The crystal structure consists of a centrosymmetric [Ni^{II}₄-(L-2H)(H₂O)₆(CH₃CH₂OH)₂]⁶⁺ cationic core with six nitrate counteranions. Two phenoxide moieties are connected through the C12–C12A bond (A: 1-x, -y, -z), forming a tetranuclear complex. In the half-structure, Ni1-N1-C5-C6-N2, Ni1-N2-C7-C8-O1, Ni2-N3-C21-C22-N4, and Ni2-N4-C23-C24-O3 form four five-membered chelate rings. The interior angles of these rings are in the range 80.99(13)-117.0(4)°, and all of the N-Ni-N and N-Ni-O angles in the rings are smaller than 90°. Two adjacent Ni atoms are bridged by the phenoxide oxygen, and the bimetallic centers are linked by the rigid biphenol ligand. In contrast to its dinuclear analogue, which has two identical Ni^{II} centers,^[19] the coordination environments, bond lengths, and angles of the two adjacent Ni atoms are somewhat different from each other in complex 1. Ni1 is coordinated by a pyridine nitrogen atom (N1), an amine nitrogen atom (N2), an appended alcoholic oxygen atom (O1), a bridging phenoxide oxygen atom (O2), a water oxygen atom (O4), and an alcoholic oxygen atom (O5), constituting a slightly distorted octahedral geometry. In contrast, although the coordination setting of Ni2 is similar to that of Ni1, ethanol does not participate in the coordination of Ni2; as an alternative, another water oxygen atom (O6) coordinates to Ni2. The Ni-O and Ni-N

FULL PAPER

bond lengths are in the ranges 2.046(3)-2.166(3) Å and 2.032(4)-2.075(4) Å, respectively, which are comparable to those previously reported for nickel(II) complexes with similar phenol-based ligands.[19,20] The distance between two adjacent nickel(II) centers 3.8025(24) Å and the bridging Ni-O-Ni angle is 133.27(14)°, which are similar to those in an analogous nickel(II) complex with one bridge (3.775 Å, 131.8°),^[21] but larger than those in a similar complex with two bridges (3.35 Å, 114°).^[20a] The distances between the non-

neighboring nickel(II) centers (Ni1…Ni1A, Ni1…Ni2A) are about 12 Å, which happen to be approximately twice the distance between two consecutive phosphate groups (6–7 Å) in a B-DNA strand.^[22] This coincidence suggests that when complex **1** interacts with phosphodiester groups in a DNA strand, the two non-neighboring nickel(II) centers may interact synchronously with two phosphoryl oxygen atoms belonging to every other phosphodiester group, thus facilitating the electrostatic interaction with DNA.

UV/Vis spectroscopic studies: The spectroscopic characteristics of L and complex 1 were first investigated by UV/Vis spectroscopy in aqueous solutions. As exhibited in Figure 2a, the ligand shows a broad absorption band in the range 250-320 nm, with λ_{max} at around 265 nm, which could be assigned to the overlapped α band of the substituted phenyl rings, as well as the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ absorption bands of the pyridyl units. This broad band displays a red shift and splitting in the spectrum of complex **1** in which λ_{max} appears at 268 nm and a moderate absorption band appears at 310 nm. The red shift of the absorption band is indicative of the coordination between L and nickel(II), particularly indicating the coordination of the phenolic oxygen (-O⁻) in the complex. Theoretically, an octahedral nickel(II) complex should give rise to four absorption bands. Of these, three strong bands result from spin-allowed transitions from the ground state ${}^{3}A_{2g}$ to the excited triplet states ${}^{3}T_{2g}$, ${}^{3}T_{1g(F)}$, and ${}^{3}T_{1g(P)}$, and a weak band arises from the spin-forbidden transition to the singlet state ${}^{1}E_{g}$, which is often observed as a shoulder of the ${}^{3}A_{2g} \rightarrow$ ${}^{3}T_{2g}$ transition band at about 800 nm.^[23] In Figure 2b, two broad absorption bands are observed in the regions 550-700 nm and 950-990 nm, respectively. The former may be attributed to the ${}^{3}A_{2g} \rightarrow {}^{3}T_{1g(F)}$ transition, whereas the latter may be assigned to the ${}^{3}A_{2g} \rightarrow {}^{3}T_{2g}$ transition. The ${}^{3}A_{2g} \rightarrow$ ${}^{3}T_{1g(P)}$ transition that would be expected to appear at around 360 nm is not observed in the spectrum, which may be due to superposition of the band by ligand-to-metal chargetransfer transitions. The characteristic bands and absorption intensities of complex 1 remained unchanged for more than



Figure 2. UV/Vis spectra of the ligand $(35 \,\mu\text{M}, 5\% \text{ ethanol})$ and complex 1 $(35 \,\mu\text{M})$ in aqueous solution (a), and enlargements of the absorption bands in the range 500–1000 nm (b); solid line (ligand), dotted line (complex 1).

one month, suggesting that the complex is quite stable in aqueous solution.

Interaction between DNA and complex 1: The interaction of DNA with complex **1** was studied by UV/Vis, fluorescence, and circular dichroism spectroscopies. Figure 3 shows



Figure 3. UV/Vis spectra of CT-DNA (0.11 mM) with complex 1 in buffer (5 mM Tris-HCl/50 mM NaCl, pH 7.3) at different [complex]/[DNA] molar ratios.

www.chemeurj.org

the variations in the UV/Vis spectrum of calf thymus (CT)-DNA in response to titration with complex **1**. At low complex to DNA molar ratios (0.01–0.05), only a moderate decrease in the absorbance of CT-DNA at 260 nm was observed. A more significant decrease was observed when the molar ratio of complex to DNA reached 0.1, suggesting that a considerable amount of free DNA had been condensed by the complex. When the molar ratio was increased to 0.2, DNA deposits were observed after centrifugation of the reaction mixture.

Ethidium bromide (EB) is a cationic dye that displays a significant increase in fluorescence when it intercalates into DNA. The enhancement of fluorescence upon intercalation of EB between base pairs of the double helix indicates the presence of native DNA in solution, and also provides the basis for DNA staining in gel electrophoresis (see the Experimental Section). Competitive displacement of EB from DNA into solution by positively charged species can result in quenching of the fluorescence.^[24] In this way, the interaction between DNA and complex **1** was investigated by means of an EB displacement assay. As shown in Figure 4,



Figure 4. Fluorescence changes of the DNA–EB system ([DNA]=[EB]= 5×10^{-5} M) upon addition of aliquots of complex 1 (3×10^{-4} M) in buffer (5 mM Tris-HCl/50 mM NaCl, pH 7.3). The inset shows the fluorescence variations (%) with the molar ratio of [complex 1]/[DNA–EB] at 599 nm.

upon addition of complex 1 to a DNA-EB solution, the fluorescence intensity decreased dramatically. A great mass of EB was expelled by the complex, and the fluorescence intensity stopped decreasing when the molar ratio of complex 1 to DNA reached 0.1. At this point, the fluorescence had been quenched to about 15% of its original intensity (inset in Figure 4). In the present case, the displacement of EB by the multivalent complex cations results from charge neutralization on the DNA backbone, which is accompanied by the collapse of linear DNA to a compact configuration without a dramatic change in the local structure.^[25] This is a highly cooperative process. Moreover, a decrease in DNA flexibility because of the condensation can shift the binding equilibrium of EB towards the solution phase.^[26] The results indicate that complex 1 interacts strongly with DNA and exerts a remarkable impact on its configuration.

FULL PAPER

Circular dichroism (CD) spectroscopy was further used to investigate the global changes in DNA conformation induced by complex **1**. As shown in Figure 5, the CD spectrum



Figure 5. Circular dichroism spectra of CT-DNA (0.1 mM) in the presence of complex **1** at different [complex]/[DNA] molar ratios.

of CT-DNA features a positive band at 275 nm due to base stacking and a negative band at 245 nm due to the helicity, which are characteristic of B-DNA.^[27] The ensuing spectra only displayed a moderate change in ellipticity for both the positive and negative bands when a small amount of complex 1 ([complex 1]/[DNA] = 0.01-0.05) was added to DNA, suggesting that the interaction between complex 1 and CT-DNA is electrostatic in nature.^[28] The CD spectra displayed an abrupt decrease when this ratio was increased to 0.1, indicating that a large amount of DNA had been induced to adopt its condensed form by complex 1. When the ratio was further increased to 0.4, both the positive and negative bands of CT-DNA almost disappeared, indicating the disappearance of the relaxed DNA and the formation of DNA condensate. The influence of the ligand on the conformation of DNA was also evaluated by CD spectroscopy. As Figure S1 (in the Supporting Information) shows, in contrast to the case of complex 1, at a [ligand]/[DNA] molar ratio of 0.4, the ellipticity of both the positive and negative bands remained nearly unchanged compared with that of free DNA. The results demonstrate that although complex 1 efficiently interacts with DNA and induces its condensation at relatively low [complex]/[DNA] molar ratios, the ligand hardly exerts any influence on the conformation of DNA.

Gel electrophoresis assay: The DNA condensation induced by complex 1 at different concentrations was tested by an agarose gel electrophoresis assay, which reflects the size/ charge ratio of the relevant DNA particles.^[29] Retardation of the DNA bands in the gel is an indication of a decrease in the negative charge on the plasmid DNA and the formation of large-sized DNA particles.^[30] As shown in Figure 6, the delay of DNA migration became increasingly evident as the concentration of complex 1 was increased from 5 to 50 μ M. Most DNA was confined to the gel slot when the con-



Figure 6. Agarose gel electrophoresis patterns of pBR322 plasmid DNA (200 ng) after incubation with different concentrations of complex 1 in buffer (50 mM Tris-HCl/50 mM NaCl, pH 7.3) at 37 °C for 2 h. Lane 1, DNA control; lanes 2–13, DNA + complex 1 (5, 10, 15, 17.5, 20, 25, 27.5, 30, 40, 50, 60, and 70 μ M, respectively).

centration of **1** reached $60 \ \mu\text{M}$. The retardation suggests that the negative charges on the DNA backbone were neutralized by the positive complex ion, and that large-sized DNA particles were formed due to a reduction of electrostatic repulsions, which would impede the migration of DNA in the gel. The results demonstrate that low concentrations of complex **1** can induce the condensation of supercoiled DNA.

Atomic force microscopy: DNA deposits on an unmodified mica surface formed from an aqueous solution (50 mM Tris-HCl, 100 mM NaCl, pH 7.3) of free DNA or DNA plus complex 1 were observed by AFM. Figure 7 shows typical AFM



Figure 7. AFM images of free supercoiled pBR322 DNA (a) and condensed DNA induced by complex 1 at 5 μ M, 2 h (b), 8 μ M, 3.5 h (c), and 10 μ M, 4 h (d) at 37 °C on the surface of mica. Scale bars: a) and b) 200 nm; c) 500 nm, c) 1 μ m.

images reflecting the morphological changes of supercoiled pBR322 DNA under different conditions. In the absence of complex **1**, DNA existed as relaxed circles with slight twisting of the strands (Figure 7a), which is characteristic of uncondensed DNA. In the presence of 5 μ M complex **1**, particles of condensed DNA began to appear, coexisting with free circular DNA (Figure 7b). When the concentration of complex **1** reached 8 μ M, the largest size of the particles increased to around 500 nm, while some amount of DNA was still in free form (Figure 7c). The supercoiled DNA was entirely induced into nanoparticles when the concentration of complex **1** reached 10 μ M (Figure 7d). The condensed DNA particles were nearly globular and their mean size was about

CHEMISTRY

500 nm. The AFM images clearly indicate that the condensation of DNA can be induced by concentrations of complex 1 of less than 10 μ M.

Dynamic light scattering: The size of the condensed DNA particles in solution induced by complex **1** was further investigated using dynamic light scattering (DLS). This measurement does not affect the electric field relevant to the DNA condensation. The distribution of hydrodynamic diameters of the DNA particles measured at a scattering angle of 90° is shown in Figure 8. The apparent mean diameter of the



Figure 8. Hydrodynamic diameter distribution of the DNA particles determined by DLS at a scattering angle of 90° and 25 °C. The concentrations of DNA and complex 1 were 20 μ gmL⁻¹ (3×10⁻⁵ Mbp) and 10 μ M, respectively.

condensed DNA particles was about 1000 nm, which is larger than that determined by AFM in the dry state. We chose 10 μ m of complex **1** to carry out the experiment because at this concentration DNA was completely condensed. Consistent with the AFM observations, the DLS results also suggested the existence of condensed DNA particles in solution.

Disassembly of the compact DNA: Intracellular release of DNA from its compact state is essential for efficient gene expression.^[7b,31] Various methods, such as increasing pH,^[32] reducing disulfide linkages,^[16] and using light-responsive or redox-active surfactants,^[26,33] have been used to trigger DNA dissociation from the vector. Here, we have used EDTA for the first time to disassemble the compact DNA preformed with complex **1**. The release process was first examined by CD spectroscopy. As shown in Figure 9, after incubation with EDTA, both the positive and negative bands of the CT-DNA spectrum displayed an increase in ellipticity, suggesting that EDTA can unpack the compact DNA induced by complex **1**, and that the amount of relaxed DNA increases with the incremental addition of EDTA.

An agarose gel electrophoresis assay was carried out to verify the CD results. Figure 10 shows the electrophoresis patterns of plasmid DNA after reaction with different concentrations of complex 1 and subsequent incubation with EDTA. In contrast to the observations in Figure 6, neither the retardation of the DNA bands in the agarose gel nor the confinement of DNA in the gel slot was observed in this experiment, even when the concentration of complex 1 was increased to 270 μ M. The results demonstrate that the compact DNA was completely restored to the relaxed form in the



Figure 9. CD spectra of preformed compact CT-DNA (0.1 mm DNA+ 0.02 mm complex 1) in the presence of EDTA at different [EDTA]/[complex] molar ratios after incubation at 37 °C and pH 7.3 for 1 h. The dashed line shows the spectrum of CT-DNA (0.1 mm) in loose form.



Figure 10. Agarose gel electrophoresis patterns of pBR322 plasmid DNA (200 ng) after reaction with complex **1** in buffer (50 mм Tris-HCl/50 mм NaCl, pH 7.3) at 37 °C for 2 h and addition of EDTA. Lane 1, DNA control; lanes 2–13, DNA+complex **1** (1, 3, 6, 10, 15, 20, 25, 30, 60, 120, 180, and 270 µм, respectively)+EDTA (3 mм).

presence of EDTA, and hence further confirm that the DNA condensation induced by complex 1 is reversible under certain conditions.

The release of DNA from the compact state by EDTA may be attributed to two factors. On the one hand, anionic EDTA may react with the condensed DNA by neutralizing the positive charge on complex 1, which would weaken the attraction between 1 and DNA, and enhance the repulsion between DNA segments, resulting in the release of DNA from the vector. This hypothesis was borne out by a zeta-potential assay, as described in the following section. On the other hand, as a strong chelating agent, EDTA may remove nickel(II) from complex 1 through an ion-exchange reaction, which would decompose complex 1 and eliminate the electrostatic attraction between 1 and DNA, leading to the dissociation of DNA from the carrier. This assumption has been verified by ESI-MS (Figure S2 in the Supporting Information), with a peak at m/z 394.08 being attributable to $[Na_2 \cdot EDTA - H + Ni]^+$ (C₁₀H₁₃N₂O₈Na₂Ni, calcd 393.88), suggesting that EDTA could grab a nickel(II) ion from complex 1 mingled with compact DNA. These CD and electrophoresis results provide some detailed information about the mechanism of DNA condensation mediated by complex 1.

Zeta potential: Zeta (ζ) potential gives an indication of the surface charge on a particulate species, which can profound-

14186 -

FULL PAPER

ly affect the aggregation and distribution of the particles.^[34] Table 2 presents the zeta potentials of DNA particles condensed at different concentrations of complex **1**. As the

Table 2. Zeta potentials of the condensed DNA particles recorded in the presence of complex **1** at different concentrations before and after the addition of EDTA.

Complex 1 [µм]	Zeta potential [mV]		
5	12.6		
8	15.8		
10	21.0		
10+EDTA (100 µм)	-29.2		

ratio of complex to DNA was increased, the positive surface charges of the DNA particles also increased; thus, the ζ potential increased with increasing concentration of complex **1**. A large surface charge can stabilize the DNA particles and prevent them from aggregating by electrostatic repulsion. On the other hand, an increase in the amount of positive complex ion can enhance its interaction with the negatively charged DNA and facilitate the DNA condensation. In the presence of EDTA, the ζ potential was seen to decrease significantly, suggesting that the positive surface charges were neutralized by the anionic EDTA. Further, the results may imply that the DNA condensation was reversed and that the negatively charged DNA was dissociated from the compact particles.

Cleavage property: The DNA cleavage property of complex **1** was studied using supercoiled pUC19 plasmid DNA by gel electrophoresis in a buffer (50 mm Tris-HCl/50 mm NaCl, pH 7.3) under physiologically relevant conditions. Figure 11



Figure 11. Agarose gel electrophoresis patterns of pBR322 plasmid DNA (200 ng) after incubation with ascorbic acid (1 mM) and complex 1 in buffer (50 mM Tris-HCl/50 mM NaCl, pH 7.3) at 37 °C for 2 h. Lane 1, DNA control; lane 2, DNA+Vc; lanes 3–10, DNA+Vc+complex 1 (0.5, 1, 2.5, 5, 10, 20, 40, and 60 μ M, respectively).

shows the electrophoresis results for DNA at increasing concentrations of complex **1** in the presence of ascorbic acid (1 mM). The supercoiled DNA (Form I) remained almost unchanged in the tested concentration range (0.5–60 μ M) as compared with the control. Therefore, this octahedral nickel(II) complex has no cleavage activity towards DNA.

Cytotoxicity: Low toxicity is a prerequisite for potential gene vectors. The cytotoxic profile of complex **1** was thus

tested in vitro against the human hepatocarcinoma cell line HepG2, the human non-small-cell lung cancer cell line A549, the human renal epithelial cell line 293T, the rat pheochromocytoma cell line PC12, and the human breast cancer cell line MCF-7. As presented in Figure 12, complex



Figure 12. Cytotoxicity of complex **1** towards HepG2, A549, 293T, PC12, and MCF-7 cell lines at concentrations of 1 and 100 μM, respectively, after incubation at 37 °C for 72 h.

1 did not exhibit obvious cytotoxicity towards the HepG2, 293T, PC12, or MCF-7 cell lines at 1 μ M, and only showed a weak toxicity towards A549 cells. Even when the concentration of complex 1 was increased to 100 μ M, the viabilities of each of the cell lines were still above 65%. The results indicate that the cytotoxicity of complex 1 is suitably low. Since the concentration of complex 1 needed for the DNA condensation is far below 100 μ M, the toxicity of this complex to organisms might be negligible in biological settings.

Conclusion

A novel tetranuclear nickel(II) complex has been designed as a potential nonviral vector for gene therapy. Low concentrations of the complex are effective in condensing DNA into nanoparticles through electrostatic interactions. The high positive charge carried by the complex ion is the major factor determining the efficiency of DNA condensation. EDTA and CD spectroscopy have been used for the first time to examine the disassembly of compact DNA in vitro. The condensed DNA can be unpacked by EDTA either through charge neutralization or ion-exchange reaction, or both, which opens up a new path for the release of confined DNA in gene delivery. Most notably, this octahedral complex exhibits no DNA cleavage activity under physiologically relevant conditions, thus ensuring the integrity of DNA during the condensation. With additional excellent properties in terms of biocompatibility and water solubility, the complex meets the various requirements of a gene vector. Studies of the transfection efficiency and gene expression level of the complex in cellular and animal models are ongoing.

Experimental Section

Materials and methods: Solvents, such as ethanol, methanol, and diethyl ether were all of analytical grade and were used as received. Calf thymus DNA (CT-DNA), pBR322 plasmid DNA, tris(hydromethyl)aminomethane (Tris), and ethidium bromide (EB) were purchased from Sigma. Ethylenediaminetetraacetic acid (EDTA) disodium salt was purchased from Alfa Aesar. Ultrapure milli-Q water was used in all experiments. IR spectra (from samples in KBr pellets) were recorded in the range 4000-500 cm⁻¹ on a Bruker VECTOR22 spectrometer. Elemental analysis was performed on a Perkin-Elmer 240C analytical instrument. UV/ Vis absorption spectra were determined on a Shimadzu UV 3600 UV/Vis spectrophotometer using solutions in 1.0 cm quartz cuvettes. CD spectra were acquired on a Jasco J-810 automatic recording spectropolarimeter. ¹H and ¹³C NMR spectra were recorded at 298 K on a Bruker DRX-500 spectrometer using standard pulse sequences. Electrospray mass spectra were recorded using an LCQ Fleet electron-spray mass spectrometer (Thermo Scientific). X-ray crystallographic raw data were collected on a Bruker SMART APEX CCD diffractometer using graphite-monochromatized Mo_{Ka} radiation ($\lambda = 0.71073$ Å) at 291(2) K. CCDC-707620 contains the crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif. Fluorescence spectra were recorded on a Perkin-Elmer LS55 luminescence spectrometer using a 1 cm cell. Hydrodynamic diameters were determined using a BI-200SM dynamic light-scattering apparatus (DLS, Brookhaven Instruments Co., Holtsville, NY). Zeta potentials were measured on a Malven Nano-Z instrument. The concentrations of CT-DNA are expressed with respect to nucleotides and were determined by UV spectroscopy at 260 nm, taking $6600 \,\mathrm{m^{-1} \, cm^{-1}}$ as the molar absorption coefficient.

Synthesis of the ligand: The intermediate 3,3',5,5'-tetrakis(hydroxymethyl)biphenyl-4,4'-diol was prepared according to a literature method with some modifications.^[35] Cold aqueous KOH solution (6%, 100 mL) was added to biphenyl-4,4'-diol (5.0 g, 26.8 mmol) and the resulting solution was stirred at room temperature for 20 min. An aqueous solution of formaldehyde (37%, 9 mL) was then added dropwise to the above solution over 2 h. After reacting for 15 days at room temperature, the resulting solution was neutralized with dilute hydrochloric acid to form a precipitate, which was collected and redissolved in ethanol. The product was purified by column chromatography (silica gel; EtOAc/EtOH, 6:1, v/v) and was obtained as a white powder in 30% yield. ¹H NMR (500 MHz, $[D_6]DMSO, 25$ °C): $\delta = 8.55$ (s, 2H; OH), 7.40 (s, 4H; Ar), 4.60 ppm (s, 8H; CH₂); ¹³C NMR (125 MHz, [D₆]DMSO, 25 °C): $\delta = 59.84$, 123.86, 129.01, 131.96, 151.01 ppm; ESI (MS): m/z: calcd for $C_{16}H_{18}O_6$ (M): [M-H]⁻: 305.11; found: 305.17. HCl gas, after drying by passage through concentrated H_2SO_4 , was passed through a suspension of 3,3',5,5'-tetrakis(hydroxymethyl)biphenyl-4,4'-diol (2.50 g, 8.17 mmol) in ethyl acetate (50 mL). When the reaction solution became clear and yellow, it was stirred under a flow of dry HCl gas at room temperature for 2 h. After removing the solvent under reduced pressure, the intermediate 3,3',5,5'tetrakis(chloromethyl)biphenyl-4,4'-diol was obtained in 55% yield by recrystallizing the crude product from hexane and ethyl acetate. ¹H NMR (500 MHz, (CD₃)₂CO, 25 °C): $\delta = 4.88$ (s, 8H; CH₂), 7.69 ppm (s, 4H; Ph); ¹³C NMR (125 MHz, (CD₃)₂CO, 25 °C): $\delta = 42.64$, 126.88, 130.23, 132.78, 153.53 ppm. A solution of 3,3',5,5'-tetrakis(chloromethyl)biphenyl-4,4'-diol (0.62 g, 1.63 mmol) and N,N-diisopropylethylamine (DIEA) in EtOAc/CHCl3 (10 mL, 1:1, v/v) was cooled to 0°C, whereupon 2-pyridylmethyl-2-hydroxyethylamine was added dropwise.[36] The resulting mixture was stirred at room temperature, and the reaction was monitored by thin-layer chromatography until it had reached completion. The solvent was removed from the mixture under reduced pressure and the residue was diluted with CHCl3. The solution obtained was washed several times with brine, dried with anhydrous Na₂SO₄, and concentrated. The crude product was purified by column chromatography (CHCl₃/CH₃OH, 8:1, v/ v) to afford yellow oily 3,3',5,5'-tetrakis{[(2-hydroxyethyl)(pyridin-2-ylmethyl)amino]methyl}biphenyl-4,4'-diol (L) in a yield of 20%. ¹H NMR $(500 \text{ MHz}, \text{CD}_3\text{OD}, 25 \,^{\circ}\text{C}): \delta = 3.03 \text{ (s, 8H; CH}_2), 3.80 \text{ (s, 8H; CH}_2), 4.14$ (s, 8H; CH₂), 4.20 (s, 8H; CH₂), 7.31–7.33 (m, 4H; Py), 7.38 (s, 4H; Ph), 7.42–7.43 (d, 4 H; Py), 7.76–7.79 (m, 4 H; Py), 8.52–8.53 ppm (d, 4 H; Py); ¹³C NMR (125 MHz, CD₃OD, 25 °C): δ =55.64, 56.14, 57.13, 58.05, 120.91, 123.31, 124.02, 128.87, 131.06, 137.74, 148.76, 154.48 ppm; ESI (MS): *m*/*z*: calcd for C₄₈H₅₈N₈O₆ (L): [L+2 H]⁺: 422.23; [L+H]⁺: 843.46; [L+Na]⁺: 865.44; found: 422.50, 843.58, 865.25.

Preparation of complex 1: Ni(NO₃)₂·6H₂O (0.14 g, 0.48 mmol) in ethanol/ H₂O (10 mL, 8:2, v/v) was added to L (0.10 g, 0.12 mmol) in ethanol (5 mL) and the mixture was stirred at room temperature for 24 h. The resulting solution was filtered and the filtrate was set aside for crystallization by slow evaporation at room temperature. Blue crystals suitable for X-ray diffraction analysis were obtained. Elemental analysis calcd (%) for C₅₂H₈₀N₁₄Ni₄O₃₂: C 37.90, H 4.89, N 11.90; found: C 37.81, H 4.74, N 11.81; IR (KBr pellet): $\tilde{\nu}$ =3417 (brm), 2921 (m), 1608 (m), 1460 (m), 1394 (s), 1105 (m), 1026 (m), 877 (s), 767 (s), 609 cm⁻¹ (m); UV/Vis: λ_{max} (ε)=267.5 (2.15757 × 10⁴), 310 nm (1.581818 × 10⁴ dm³ mol⁻¹ cm⁻¹).

X-ray crystal diffraction: An empirical absorption correction was applied to the raw crystallographic data of complex 1 using the SADABS multiscan program.^[37] The crystal structure was solved by direct methods and refined by the full-matrix least-squares technique using the SHELXTL program package.^[38] Anisotropic thermal parameters were assigned to all non-hydrogen atoms. All hydrogen atoms were placed in calculated positions and refined using a riding model.

Circular dichroism study: CD spectra of CT-DNA (0.1 mM) in the presence or absence of complex **1** were collected after incubation in buffer (5 mM Tris-HCl/50 mM NaCl, pH 7.3). All CD experiments were performed at 25 °C and involved scanning at a speed of 10 nmmin⁻¹ from 220 to 320 nm. The buffer background was subtracted.

EB displacement assay: A solution of complex **1** was dropped into the EB–DNA ([EB]/[DNA]=1:1, [DNA]= 5×10^{-5} M) system in buffer (5 mm Tris-HCl/50 mm NaCl, pH 7.3). The mixture was allowed to equilibrate for 1 min. Fluorescence spectra with an excitation wavelength of 526 nm were recorded in the emission range 530–750 nm.

Agarose gel electrophoresis: Supercoiled pBR322 DNA (200 ng) was treated with gradient concentrations of complex 1 in buffer (50 mm Tris-HCl/50 mm NaCl, pH 7.3) until the total added volume reached 10 μ L. The mixtures were incubated at 37 °C for 2 h, and then loading buffer (36% glycerol, 0.05% xylene cyanol FF, 0.05% bromophenol blue), without or with EDTA (3 mm), was added to the solutions. The resulting solutions were loaded onto agarose gel (1%) and subjected to electrophoresis in a buffer (40 mm Tris/acetate). DNA bands were stained with EB, visualized under UV light, and photographed. The DNA cleavage experiment was carried out according to a similar method, except that ascorbic acid (1 mm) was added to the reaction system as a cleaving initiator.

Atomic force microscopy: Samples of pBR322 DNA (200 ng) were prepared and incubated with gradient concentrations of complex 1 in buffer (50 mM Tris-HCl/50 mM NaCl, pH 7.3) and the total volume was adjusted to 10 μ L. Following the digestion, each sample was applied to a mica surface and the volatiles were evaporated. The remaining salts were rinsed with ultrapure water (50 μ L) and then dried with N₂. AFM images were obtained on a Nanoscope V multimode scanning probe workstation using etched silicon nano-probes (probe model AC160 TS, Digital Instruments, Olympus) under ambient conditions. Nanoscope VII software provided by the manufacturer of the AFM instrument was used to measure the volume distributions (height, width, and length) of the DNA deposits on the mica. Five random spots in the entire area were analyzed in each case.

Dynamic light-scattering analysis: DNA solutions $(20 \ \mu g m L^{-1})$ were mixed with complex **1** (10 μ M) in Tris-HCl buffer (pH 7.3), and each mixture (2 mL) was transferred to a standard quartz cuvette. The samples were allowed to stand for 2 min at room temperature before measurement. Dynamic light scattering was used to determine the size distribution of the DNA particles condensed by complex **1**. The scattering angle was set at 90°.

Zeta potential measurement: DNA solution ($6 \mu g m L^{-1}$) was mixed with complex **1** (5, 8, 10 μ M, respectively) or with **1** in the presence of EDTA (100 μ M). Each mixture was incubated at 37 °C for 60 min. Zeta potential

14188 -

FULL PAPER

was measured in aqueous solution at pH 7.1. The mean of triplicate measurements was taken as the final result.

Cytotoxicity assay: The cytotoxicities of complex 1 towards HepG2, A549, 293T, PC12, and MCF-7 cell lines were tested by MTT assays (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).^[39] Cells of each type were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with fetal bovine serum (10%, v/v), streptomycin (0.1 mg mL⁻¹), and penicillin (100 U mL⁻¹) in a humid atmosphere containing 5% CO2 at 37°C. The cell lines were seeded, respectively, in 96-well plates at 5×10^3 cells per well in DMEM medium, incubated overnight, and then treated thrice with fresh medium containing different concentrations of complex 1. The cells were incubated at 37°C under 5% CO_2 for 72 h and then incubated with MTT solution (5 $mg\,mL^{^{-1}}\!\!,\,10\,\mu L)$ in PBS buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH₂PO₄ per liter) for 4 h. DMSO (150 µL) was added to each well after the medium had been removed. The absorbance of the purple formazan was recorded at 490 nm using an ELISA plate reader. The viability of the cells was calculated based on the data of three parallel tests.

Acknowledgements

We are grateful to the financial support from the National Natural Science Foundation of China (Nos.: 20631020, 90713001, 20721002, and 30870554) and the Natural Science Foundation of Jiangsu Province (BK2008015).

- a) D. Ferber, Science 2001, 294, 1638–1642; b) Y. Liu, Z. L. Yu, Y. M. Zhang, D. S. Guo, Y. P. Liu, J. Am. Chem. Soc. 2008, 130, 10431–10439; c) C. E. Pedraza, D. C. Bassett, M. D. McKee, V. Nelea, U. Gbureck, J. E. Barralet, Biomaterials 2008, 29, 3384–3392.
- [2] I. W. Hamley, V. Castelletto, Angew. Chem. 2007, 119, 4524–4538; Angew. Chem. Int. Ed. 2007, 46, 4442–4455.
- [3] V. A. Bloomfield, Biopolymers 1997, 44, 269-282.
- [4] A. Rémy-Kristensen, J. P. Clamme, C. Vuilleumier, J. G. Kuhry, Y. Mély, *Biochim. Biophys. Acta* 2001, 1514, 21–32.
- [5] M. A. Mintzer, E. E. Simanek, Chem. Rev. 2009, 109, 259-302.
- [6] N. Korolev, N. V. Berezhnoy, K. D. Eom, J. P. Tam, L. Nordenskiöld, *Nucleic Acids Res.* 2009, 37, 7137–7150.
- [7] a) J. P. Behr, Acc. Chem. Res. 1993, 26, 274–278; b) V. Vijayanathan, T. Thomas, T. J. Thomas, Biochemistry 2002, 41, 14085–14094; c) K. Fant, E. K. Esbjörner, P. Lincoln, B. Nordén, Biochemistry 2008, 47, 1732–1740.
- [8] a) D. Putnam, Nat. Mater. 2006, 5, 439–451; b) H. K. Nguyen, P. Lemieux, S. V. Vinogradov, C. L. Gebhart, N. Guérin, G. Paradis, T. K. Bronich, V. Y. Alakhov, A. V. Kabanov, Gene Ther. 2000, 7, 126– 138; c) T. H. Kim, H. L. Jiang, D. Jere, I. K. Park, M. H. Cho, J. W. Nah, Y. J. Choi, T. Akaike, C. S. Cho, Prog. Polym. Sci. 2007, 32, 726–753; d) J. H. Yang, P. Zhang, L. Tang, P. Sun, W. G. Liu, P. Sun, A. J. Zuo, D. C. Liang, Biomaterials 2010, 31, 144–155.
- [9] S. K. Kim, K. M. Park, K. Singha, J. Kim, Y. Ahn, K. Kim, W. J. Kim, Chem. Commun. 2010, 46, 692–694.
- [10] a) A. Rodger, K. J. Sanders, M. J. Hannon, I. Meistermann, A. Parkinson, D. S. Vidler, I. S. Haworth, *Chirality* 2000, *12*, 221–236; b) S. He, P. G. Arscott, V. A. Bloomfield, *Biopolymers* 2000, *53*, 329–341; c) B. I. Kankia, V. Buckin, V. A. Bloomfield, *Nucleic Acids Res.* 2001, *29*, 2795–2801; d) C. C. Conwell, I. D. Vilfan, N. V. Hud, *Proc. Natl. Acad. Sci. USA* 2003, *100*, 9296–9301.
- [11] X. G. Meng, L. Liu, C. S. Zhou, L. Wang, C. L. Liu, *Inorg. Chem.* 2008, 47, 6572–6574.
- [12] a) E. Musatkina, H. Amouri, M. Lamoureux, T. Chepurnykh, C. Cordier, *J. Inorg. Biochem.* **2007**, *101*, 1086–1089; b) B. Sun, J. X. Guan, L. Xu, B. L. Yu, L. Jiang, J. F. Kou, L. Wang, X. D. Ding, H. Chao, L. N. Ji, *Inorg. Chem.* **2009**, *48*, 4637–4639.

- [13] J. Pelta, F. Livolant, J. L. Sikorav, J. Biol. Chem. 1996, 271, 5656– 5662.
- [14] D. Lechardeur, G. L. Lukacs, Curr. Gene Ther. 2002, 2, 183-194.
- [15] a) D. V. Schaffer, N. A. Fidelman, N. Dan, D. A. Lauffenburger, *Biotechnol. Bioeng.* 2000, 67, 598–606; b) M. L. Read, K. H. Bremner, D. Oupický, N. K. Green, P. F. Searle, L. W. Seymour, *J. Gene Med.* 2003, 5, 232–245.
- [16] Y. Z. You, Z. Q. Yu, M. M. Cui, C. Y. Hong, Angew. Chem. 2010, 122, 1117–1120; Angew. Chem. Int. Ed. 2010, 49, 1099–1102.
- [17] a) D. G. Barceloux, *Clin. Toxicol.* **1999**, *37*, 239–258; b) M. J. Maroney, *Curr. Opin. Chem. Biol.* **1999**, *3*, 188–199; c) P. E. Carrington, P. T. Chivers, F. Al-Mjeni, R. T. Sauer, M. J. Maroney, *Nat. Struct. Biol.* **2003**, *10*, 126–130.
- [18] X. Y. Chen, S. E. Rokita, C. J. Burrows, J. Am. Chem. Soc. 1991, 113, 5884–5886.
- [19] J. W. Chen, B. Liu, X. Q. Chen, Y. Z. Li, Acta Crystallogr. Sect. C 2007, 63, m42-m44.
- [20] a) H. Sakiyama, T. Suzuki, K. Ono, R. Ito, Y. Watanabe, M. Yamasaki, M. Mikuriya, *Inorg. Chim. Acta* 2005, *358*, 1897–1903; b) A. Asokan, B. Varghese, A. Caneschi, P. T. Manoharan, *Inorg. Chem.* 1998, *37*, 228–232.
- [21] M. A. de Brito, A. J. Bortoluzzi, A. Greatti, A. S. Ceccato, A. C. Joussef, S. M. Drechsel, *Acta Crystallogr. Sect. C* 2000, 56, 1188–1190.
- [22] a) C. Sissi, P. Rossi, F. Felluga, F. Formaggio, M. Palumbo, P. Tecilla, C. Toniolo, P. Scrimin, *J. Am. Chem. Soc.* 2001, *123*, 3169–3170;
 b) Y. An, M. L. Tong, L. N. Ji, Z. W. Mao, *Dalton Trans.* 2006, 2066–2071.
- [23] C. Ochs, F. E. Hahn, T. Lügger, Eur. J. Inorg. Chem. 2001, 1279– 1285.
- [24] V. A. Izumrudov, M. V. Zhiryakova, A. A. Goulko, *Langmuir* 2002, 18, 10348–10356.
- [25] a) G. S. Manning, *Biopolymers* **1980**, *19*, 37–59; b) G. S. Manning, *Biopolymers* **1981**, *20*, 1261–1270.
- [26] A.-L. M. Le Ny, C. T. Lee, Jr., Biophys. Chem. 2009, 142, 76-83.
- [27] W. C. Johnson, in *Circular Dichroism: Principles and Applications* (Eds.: K. Nakanishi, N. Berova, R. W. Woody), VCH, Weinheim, **1994**, pp. 523–540.
- [28] S. P. Tang, L. Hou, Z. W. Mao, L. N. Ji, Polyhedron 2009, 28, 586– 592.
- [29] C. F. Ke, S. Hou, H. Y. Zhang, Y. Liu, K. Yang, X. Z. Feng, Chem. Commun. 2007, 3374–3376.
- [30] S. J. Sun, W. G. Liu, N. Cheng, B. Q. Zhang, Z. Q. Cao, K. D. Yao, D. C. Liang, A. J. Zuo, G. Guo, J. Y. Zhang, *Bioconjugate Chem.* 2005, 16, 972–980.
- [31] L. de Laporte, J. C. Rea, L. D. Shea, Biomaterials 2006, 27, 947-954.
- [32] N. Makita, K. Yoshikawa, *Biophys. Chem.* 2002, 99, 43–53.
- [33] M. E. Hays, C. M. Jewell, D. M. Lynn, N. L. Abbott, *Langmuir* 2007, 23, 5609–5614.
- [34] R. M. Xing, X. Y. Wang, C. L. Zhang, Y. M. Zhang, Q. Wang, Z. Yang, Z. J. Guo, J. Inorg. Biochem. 2009, 103, 1039–1044.
- [35] W. F. Veldhuyzen, P. Pande, S. E. Rokita, J. Am. Chem. Soc. 2003, 125, 14005–14013.
- [36] J. W. Chen, X. Y. Wang, Y. G. Zhu, J. Lin, X. L. Yang, Y. Z. Li, Y. Lu, Z. J. Guo, *Inorg. Chem.* 2005, 44, 3422–3430.
- [37] SADABS, Version 2.03, G. M. Sheldrick, University of Göttingen (Germany), 2002.
- [38] a) SHELXS97 and SHELXL97, G. M. Sheldrick, University of Göttingen (Germany), 1997; b) SHELXTL, Version 6.10, G. M. Sheldrick, Bruker AXS Inc., Madison, Wisconsin, 2002.
- [39] M. B. Hansen, S. E. Nielsen, K. Berg, J. Immunol. Methods 1989, 119, 203-210.

Received: May 26, 2010 Published online: October 22, 2010