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一种新型磁性纳米基因载体的制备与表征

郝丽娟 李双艳 韩 磊 黄 杰 常 津*
(天津大学材料科学与工作学院纳米生物技术研究所, 天津 300072)

摘要: 制备了一种新的基因载体材料——赖氨酸修饰的壳聚糖(CTS-lys)包裹的磁性纳米颗粒. 优化制备了CTS-lys 原料, 红外(IR)和核磁(^{1}H-NMR)检测结果表明壳聚糖的大量氨基被赖氨酸修饰. 通过共沉淀方法, 制备了赖氨酸修饰的壳聚糖磁性纳米颗粒(CTS-lys-MNPs). 利用透射电镜(TEM)、激光粒度分析仪、磁力计(VSM)和 X 射线衍射(XRD)对 CTS-lys-MNPs 进行了表征, 并通过 U293 细胞, 研究了 CTS-lys-MNPs 的细胞毒性. 结果表明, CTS-lys-MNPs 的平均粒径为 100 nm, 具有较好的超顺磁性和较低的细胞毒性; 在此基础上, 通过凝胶电泳实验观察了 CTS-lys-MNPs 和 DNA 的结合情况, 并通过单光子发射型计算机断层显像仪 (SPECT) 研究了 CTS-lys-MNPs 和 DNA 的复合物在动物体内跨越血脑屏障的能力. 结果表明, CTS-lys-MNPs 是一种较好的磁靶向基因载体并能成功地跨越血脑屏障.

关键词: 壳聚糖; 赖氨酸; 磁性纳米颗粒; 靶向; 基因载体; 血脑屏障
中图分类号: O648

Preparation and Characterization of a Novel Magnetic Nano-Gene Vector

HAO Li-Juan LI Shuang-Yan HAN Lei HUANG Jie CHANG Jin*
(*Institute of Nanobiotechnology, School of Materials Science and Engineering, Tianjin University, Tianjin 300072, P. R. China*)

Abstract: In recent times, as nonviral gene vectors, chitosan and its derivations have attracted many researchers' attentions. The preparation of a novel gene vector-magnetic nanoparticle coated with a novel polymer-lysine modified chitosan (CTS-lys) is described in this article. Initially, the correlation conditions of preparing CTS-lys were optimized, and it was demonstrated that lots of amines of chitosan were modified by lysine, which was indicated by infrared spectroscopy (IR) and proton nuclear magnetic resonance (^{1}H-NMR). Subsequently, through the coprecipitation method, the lysine modified chitosan magnetic nanospheres (CTS-lys-MNPs) were attained. The characterizations of nanospheres were measured by transmission electron microscopy (TEM), dynamic laser light scattering, vibrating samples magnetometer (VSM), and X-ray diffraction (XRD). The cytotoxicity of CTS-lys-MNPs was also investigated with U293 cells. The results indicated that the average size of nanospheres was about 100 nm, and they had narrower size distribution, good superparamagnetic property, and perfect crystallinity, and they also retained low toxicity. Additionally, the combination of DNA and CTS-lys-MNPs was observed *via* agarose gel electrophoresis, and the ability of complexes of CTS-lys-MNPs and DNA crossing blood-brain barrier (BBB) in rats was studied by single photon emission computed tomography (SPECT). The results showed that the gene vector was a superior material, which had the ability of targeting and avoiding the caption of BBB.

Key Words: Chitosan; Lysine; Magnetic nanospheres; Target; Gene vector; Blood-brain barrier

Gene therapy is promising for curing various inherited or acquired diseases. However, the lack of safe and efficient carri-

Received: July 30, 2007; Revised: September 3, 2007; Published on Web: October 9, 2007.
English edition available online at www.sciencedirect.com
*Corresponding author. Email: jinchang@tju.edu.cn; Tel: +8622-27401821.
国家自然科学基金(50373033), 天津市应用基础研究重点基金(05YFJZJC01001)和天津市国际合作基金项目(05YFGHHZ20070)资助

ers of DNA is a main hurdle to reach the success of gene therapy[1]. Great concern toward the safety of viral vectors makes the non-viral ones much more attractive.

In the past decades, chitosan (CTS), a naturally occurring linear cationic polysaccharide, has been widely employed as a drug/gene delivery system for wound dressing, as an anticoagulant and a scaffold for tissue engineering, owing to its biocompatibility, biodegradability, and low toxicity[2]. But as chitosan is insoluble in neutral or biological solutions and readily precipitated within a few days, it is difficult for chitosan-based self-aggregates to be widely applied to drug/gene delivery systems[3,4]. Recently, water-soluble chitosan derivatives have been used to increase their stability and decrease cytotoxicity[5,6].

Additionally, it is well known that there has been a great interest in developing and testing iron oxide nanoparticles for tumor detection and therapy in the past two decades. In brain research, nanodispersed iron oxides have been used as carriers of diagnostic and therapeutic agents for mapping the blood-brain barrier disruption, to improve tumor detection and therapy[7–9].

Here we have designed and synthesized a novel water soluble polymer (CTS-lys) and used it as a coat, to prepare magnetic microspheres. The reason that we have chosen CTS-lys as the coating agent is because it is biocompatible, biodegradable, nontoxic, and water soluble. Moreover, it also has some unique antitumor and antibacterial bioactivities[10]. Another appealing characteristic of CTS-lys is that it has more positive charges than many other chitosan derivatives, which can improve the capacity for carrying DNA.

1 Materials and methods

1.1 Materials

Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), ammonium hydroxide (5 mol·L^{-1}), hydrochloric acid (HCl), and dimethylfomamide (DMF) were obtained from local suppliers. Deionized water was used in all the steps involved in the synthesis and formulation of magnetic nanoparticles. Chitosan was obtained from Zhejiang Yuhuan Chitin Company (China). Lysine and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) were both obtained from Dingguo Biotechnology Company (China). Fluorescamine was obtained from Fluka (Switzerland). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), gel filtration were obtained from Sigma, USA. DNA particles were supplied by Tianjin Medical University (China). All the chemicals were of analytical grade and no further purification was required.

1.2 Synthesis and characterization of lysine modified chitosan

1.2.1 Synthesis of lysine modified chitosan

Typically, 0.11 g of chitosan powder was dissolved in 10 mL of deionized water, and 3 mol·L^{-1} HCl was added with the molar ratio of HCl to the amine of chitosan to be 1:10. Then lysine, EDC, and DMF were added to the solution under stirring. Polymerization was performed at constant temperature for some time. Finally, the mixture was centrifuged, and the precipitate was washed with ethanol thrice. After being purified by dialyzing in water, the solution was lyophilized, to give a light white powder.

Table 1 The correlation experiments

	m(chitosan)/g	*m*(lysine)/g	*T*/℃	Molar ratio of O/W	*t*/h	Stirring rate (r·min^{-1})
(1)	0.124	0.215	0	1:1	2	1000
(2)	0.123	0.219	20	1:1	2	1000
(3)	0.122	0.218	40	1:1	2	1000
(4)	0.125	0.214	20	1:1	4	1000
(5)	0.121	0.214	20	1:1	6	1000
(6)	0.126	0.216	20	0.5:1	2	1000
(7)	0.127	0.217	20	2:1	2	1000
(8)	0.125	0.215	20	1:1	2	800
(9)	0.124	0.216	20	1:1	2	1200

To acquire the respective experimental conditions, such as, pH, temperature, stirring rate, ratio of oil phase and water phase (O/W), the reaction time and so on, we designed correlation experiments (Table 1).

1.2.2 Analysis of the amounts of amino group of CTS-lys

Free primary amino groups of CTS-lys were determined by the fluorescamine assay as previously described[11–13]. An aliquot of 100 μL of each sample was diluted with 1.4 mL assay buffer (100 mmol·L^{-1} boric acid-NaOH, pH=7.0–9.5), and 500 μL 0.01% fluorescamine (prepared in acetone) was rapidly added. Samples were rapidly inverted four to five times and incubated at room temperature for 10 min. Fluorescence was measured using a Hitachi F-4500 Fluorescence Spectrophotometer with λ_{ex}=392 nm, λ_{em}=480 nm, and 5–10 nm slit widths. Background fluorescence was measured using the assay buffer only.

1.3 Synthesis of CTS-lys magnetic nanoparticles (CTS-lys-MNPs)

Solution A: 3.0 g CTS-lys (MW 40000) and 0.326 g ferric in 15.0 mL H_2O were passed through a filter (220 nm) and precooled on ice. Solution B: 0.150 g ferrous in 1 mL H_2O was passed through a filter (220 nm) and precooled on ice. Solution B was added dropwise into solution A under rapid stirring, followed by 5 mL 20% NaOH. Later, the solution was slowly heated to 70–90 ℃ within 1 h. The solution was kept at this temperature and stirred for another 30 min. After elimination of sodium hydroxide by dialyzing (MWCO 14000) in distilled water, CTS-lys-MNPs were separated by a magnet and the pellets were prepared using lyophilization.

1.4 Cytotoxicity assay of CTS-lys-MNPs

U293 cells (4000 cells/well) were seeded in 96-well plates. The cells were incubated for 4 h with 200 μL of complete culture medium containing CTS-lys-MNPs at different concentrations. The medium in each well was replaced with 100 μL of complete fresh medium 4 h later. After 25 μL of MTT solution in PBS (5 g·L^{-1}) was added, the cells were incubated for another 2 h. Subsequently 100 μL of the extraction buffer (20% sodium dodecyl sulfate (SDS) in 50% DMF, pH=4.7) was added to the

wells, and the cells were incubated overnight. The optical intensity was measured at 550 nm using a microplate reader (model 550, BioRad Lab, Hercules, CA). In addition, the cytotoxicity of the pure synthetic magnetites (MNPs) was evaluated. As they were being used for gene delivery, CTS-lys-MNPs would be complexed with DNA, and the complexes would generally show less toxicity than pure MNPs[14,15].

1.5 Complexes of CTS-lys-MNPs and DNA

1.5.1 Formation of the MNPs-DNA complexes

All complexes of DNA and polymer were freshly prepared before use. CTS-lys-MNP solutions were added to the DNA solutions in equal volumes, mixed by vortexing, and incubated for 30 min before use, unless otherwise stated. DNA stock solutions were diluted to 7.4. The CTS-lys-MNP stock solutions were diluted, with the same medium as mentioned earlier, to the appropriate concentration depending on the required mass ratio, and filtered through 0.22 μm.

1.5.2 Physicochemical characterization of CTS-lys-MNPs/DNA complexes

The condensation capability of CTS-lys-MNPs with DNA was evaluated using agarose gel electrophoresis[16–18]. A number of 40 μL aliquots of the complex solutions were mixed with 4 μL of loading buffer [glycerol (85%) and TAE (40 mmol·L^{-1} Tris/HCl, 1% acetic acid, 1 mmol·L^{-1} EDTA, pH=7.4) in equal parts] and loaded onto an EtBr containing 1% agarose gel. Electrophoresis was carried out at a voltage of 50 V (LKB Bromma 2197 Power Supply, Pharmacia, Freiburg, Germany) for 1 h, in a TAE running buffer solution. UV light (254 nm) detection was conducted with a Video Copy Processor.

1.6 Evaluating ability of CTS-lys-MNPs crossing BBB *in vivo*

1.6.1 ^{99m}Tc label to CTS-lys-MNPs

One milliliter of CTS-lys-MNPs in 1 mL 0.1 mol·L^{-1} phosphate buffer pH 7.4 reacted with 5 mg DTPA at room temperature for 1 h. The resultant solution was applied to a magnetic field to separate the unconjugated DTPA. 5 mg 0.5 mL $^{99m}TcO_4^-$ [25 mCi] was added to the above mixture, and then kept at room temperature for 20 min. Five milligrams of $NaBH_4$ was added to the above solution[19,20].

1.6.2 *In vivo* studies

Rats (Male Fishers, 225–250 g, which were supplied by Tianjin Medical University, China) were anesthetized with an intraperitioneal injection of a 3:2:1 (volume ratio) mixture of ketamine hydrochloride (100 g·L^{-1}), acepromazine, maleate (10 g·L^{-1}), and xylazine hydrochloride (20 g·L^{-1}), at a dose of 0.1 mg/100 g body weight. Then the rats were injected in the carotid with radio labeled CTS-lys-MNPs, about 1 mL. After being injected, the rats were kept between the poles of the magnet (1 T) for 30 min. The rats were studied by single photon emission computed tomography (SPECT).

2 Results and discussion

2.1 Synthesis and characterization of CTS-lys

The intensity of fluorescence represented the existence of amino groups in CTS-lys, which reflected the reaction degree between chitosan and lysine[10–13]. Lysine modified chitosan was obtained under different conditions as mentioned earlier.

2.1.1 Effect of pH

From Fig.1(A), it could be deducted that the intensity of the fluorescence was increased with the increase in pH, when the pH of the solution was below 5. This was because EDC played an important role during the reaction. Subsequently, with the pH of the solution increasing to 5, the activity of EDC enhanced, and more lysine reacted with chitosan, and the free amino

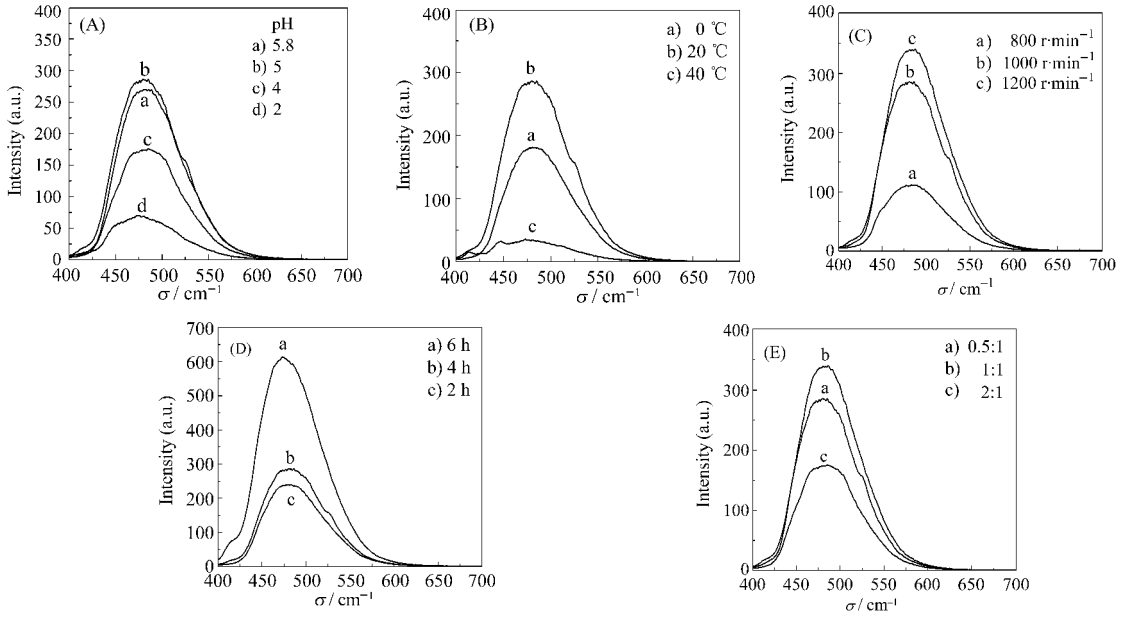


Fig.1 Influences of pH (A), temperature (B), stirring speed (C), reaction time (D), and the volume ratio of DMF/H_2O (E) on the fluorescence intensity

groups of the CTS-lys increased. Hence, the intensity of the fluorescence increased. When the pH was above 5, chitosan could have poor dissolution in the solution, which restricted the reaction. Especially when the pH was 7, the production was not water-soluble.

2.1.2 Effect of reaction temperature

Fig.1(B) shows the results of the effect of reaction temperature. The increase in the reaction temperature enhanced both the rate and degree of the reaction. All these factors would increase the extent of modification. However, the activity of EDC was limited when the temperature exceeded 40 ℃.

2.1.3 Effect of stirring rate

As seen in Fig.1(C), the intensity of fluorescence increased with the increase in stirring rate. That is, the number of free amino groups increased with the increase in stirring rate. This could be because of the fact that high stirring speed could generate powerful shearing strength, and thus enhance the reaction of lysine and chitosan. At the same time, too high speed stirring, such as, above 1200 $r \cdot min^{-1}$, was not necessary, as it would splash the reaction solution.

2.1.4 Effect of reaction time

The effect of the reaction time on synthesis of lysine and chitosan is shown in Fig.1(D). It can be seen that the intensity of fluorescence increases with the extention of reaction time. An increase in reaction time enhances the extent of reaction of lysine and chitosan, so that the reaction can be carried through completely. However, longer reaction time is not necessary when the reaction is over.

2.1.5 Effect of O/W molar rate

The effect of the molar ratio of O/W on fluorescence intensity is shown in Fig.1(E). When the molar ratio of O/W is one, the fluoresencence is the brightest. This may be because of the fact that when the molar ratio of O/W is one, lysine and chitosan can react with each other completely. When the ratio is above or below one, the solvent limits the reaction of lysine and chitosan. Therefore, the optimization molar ratio of O/W is one.

2.1.6 IR characterization of CTS-lys

IR spectra were recorded in the range of 4000–400 cm^{-1}. From Fig.2, it could be seen that bands of methylene and amide were obviously sharper than those of chitosan. The absorption bands at 1550 and 1650 cm^{-1} were because of —NH_2, and the absorptions at 3100–3000 cm^{-1} were attributed to the presence of —CH_3. It should be noted that spectrum A was particularly sharper in comparison with spectrum B, supporting the fact that most of the amino groups of chitosan were substituted by lysine.

2.1.7 ^{1}H-NMR characterization of CTS-lys

^{1}H-NMR spectrum was recorded by a JNM-A500 500 MHz spectrometer at 70 ℃ using CD_3COOD/D_2O (the proton resonates at 4.67) as a solvent. Chemical shifts (δ) were given using tetramethylsilane as the internal reference.

From Fig.3(A), the proton assignment of CTS[21–23] could be obtained as follows: 1.810 (the peak of the acetyl group, HAc), 2.933 (the peak of proton H2 of the deacetylated monomer H2D), 3.4–3.8 (the peaks of protons H2, H3, H4, H5, H6, H6′, H26), 4.92 (the peak of the proton H1 of the acetylated monomer, H1A), 4.98 (the peak of proton H1 of the deacetylated monomer, H1D). From Fig.3(B), the proton assignment of CTS-lys could be obtained as follows: 1.473 (the peak of proton H10 of CTS-lys unit H10B), 1.828 (HAc and the peak of proton H8 of CTS-lys unit, H8B), 1.204 (the peak of proton H9 of CTS-lys unit, H9B), 2.755 (the peak of proton H11 of CTS-lys unit, H11B), 2.940 (H2D and the peak of proton H8 of CTS-lys unit, H2B), 3.4–3.8 (H26 and the peak of proton H7 of CTS-lys unit, H7B), and 4.92 (H1A).

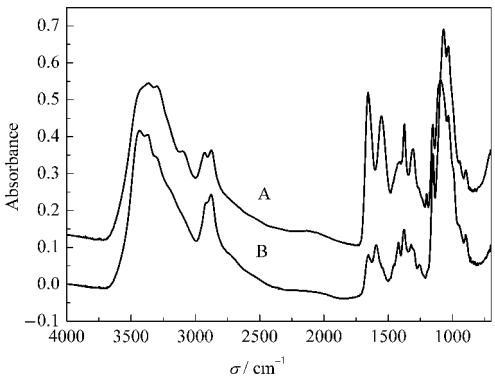


Fig.2 IR spectra of CTS-lys (A) and CTS (B)

By comparison, in Fig.3(A, B), it could be seen that one lysine side chain contained around the chitosan chain in the ^{1}H-NMR curve of CTS-lys had substituted chitosan to a large degree.

2.2 Characterization of CTS-lys magnetic nanoparticles

2.2.1 Morphology of CTS-lys magnetic nanoparticles

The morphology, average size, and size distribution of CTS-lys-MNPs were measured by JEOL-100CXII TEM, and 90 Plus/BI-MAS Multi Angle Particle Sizing Instruments (Brookhaven Instruments Co.). The results are shown in Figs.4 (A, B). The average size of CTS-lys-MNPs was about 100 nm, with well-shaped morphology and narrow size distribution.

2.2.2 Crystalline phase of CTS-lys-MNPs

As shown in Fig.4(C), the XRD powder diffraction peaks of CTS-lys-MNPs matched well with the standard reflections of Fe_3O_4. Thus, it can be confirmed that CTS-lys-MNPs consist of magnetites. It is well known that the full width of half maximum (FWHM) of the XRD pattern reflected crystallinity of the samples[24]. As shown in Fig.4(C), the CTS-lys-MNPs have perfect crystallinity for their narrow FWHM.

2.2.3 Magnetic properties of CTS-lys-MNPs

Fig.4(D) shows the magnetization (M) *versus* field (H) curve at 27 ℃ for samples of CTS-lys-MNPs. From the curve, it is observed that the remnant magnetization (M_r) value for the products is 1.259 $emu \cdot g^{-1}$, which is 3.7% of the saturation magnetization (M_s=39.2 $emu \cdot g^{-1}$). The result was perfectly comparable with that reported in the literature[25]. Thus, CTS-lys-MNPs had no remanence and no coercivity that showed superparamagnetic behavior.

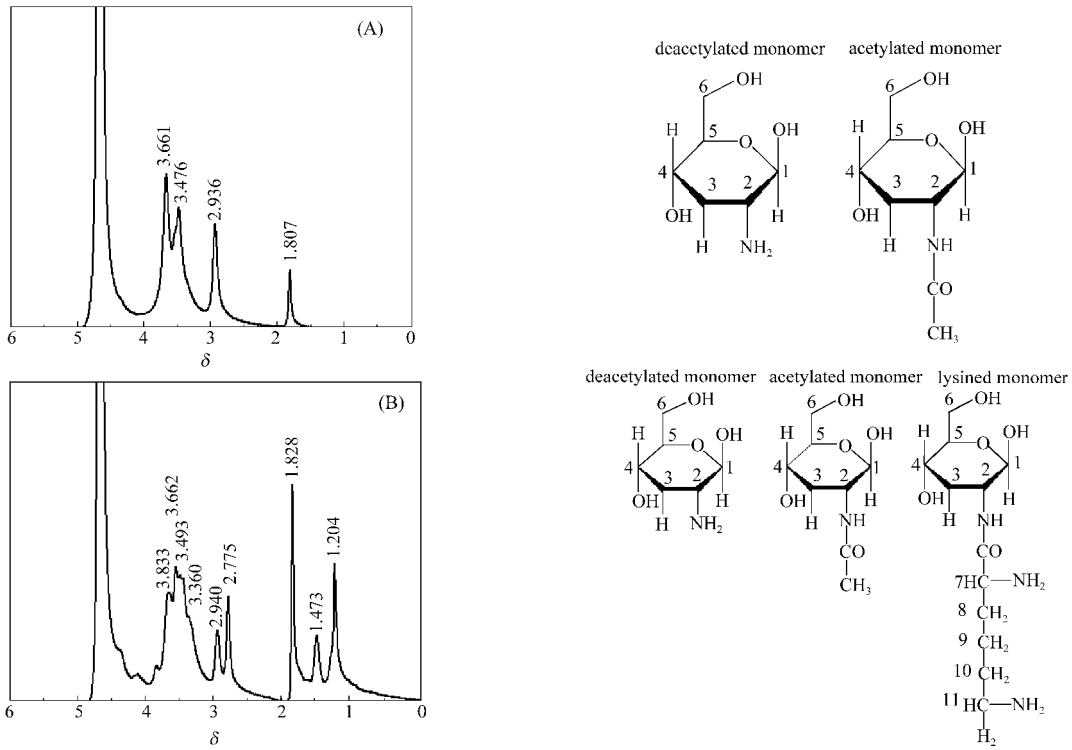


Fig.3 ^{1}H-NMR curves of CTS (A) and CTS-lys (B) and structures of monomers

2.3 Cytotoxicity of CTS-lys-MNPs

The MTT test was used to determine effects of both CTS-lys-MNPs and MNPs on metabolic activity. As shown in Fig. 5 (A), CTS-lys-MNPs affected the metabolic activity in a concentration- and time-dependent manners when they were incubated with U293 cells, in a concentration from low to high, for 1 d to 7 d. It was clear that the cytotoxicity of the CTS-lys-MNPs and MNPs decreased in relation to decreased concentration and incubation time. After 7 d, more than 55% viable cells were determined, after incubation of MNPs. However, incubation with CTS-lys-MNPs after 7 d showed that the viability of cells was still above 90%. As is known, the cytotoxicity of nanomaterial is thought to be the result of the interaction between the positive charges on the nanomaterial surfaces and the negative charges on the cell surfaces[26]. Because there were some positive charges on the surface of CTS-lys-MNPs and some hydroxyl groups on MNPs surface, weak interaction between them and cells occurred, which led to their low cytotoxicities. At the same time, the polymer surface endowed them with better biocompatibility than MNPs. In addition, the cytotoxicity of the CTS-lys-MNPs was evaluated. As they were used for gene delivery, CTS-lys-MNPs would be complexed with DNA, and the complexes generally showed less toxicity than pure MNPs.

2.4 Physicochemical characterization of CTS-lys-MNPs/DNA complexes

The condensation capability of CTS-lys-MNPs with DNA was evaluated using agarose gel electrophoresis. Fig.5(B) demonstrated that the migration of DNA was completely retarded when the mass ratio of CTS-lys-MNPs/DNA was around 1∶4. It could also be seen that the complexation was hindered slightly (mass ratio was 1∶5). The migration of plasmid DNA in the gel was retarded, as the ratio of CTS-lys-MNPs/DNA was increased above 1∶4 for all five gels, demonstrating that the CTS-lys-MNPs was capable of binding with DNA, and it was a new gene delivery system.

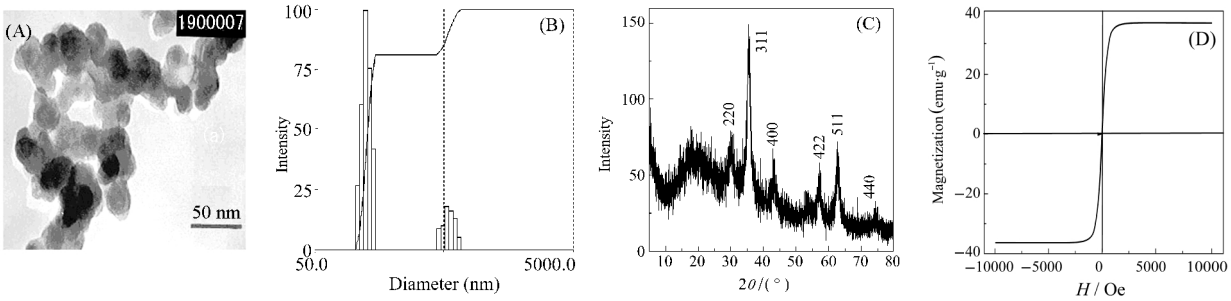


Fig.4 TEM photograph (A), size distribution (B), XRD pattern (C), and magnetization curve (D) of CTS-lys-MNPs

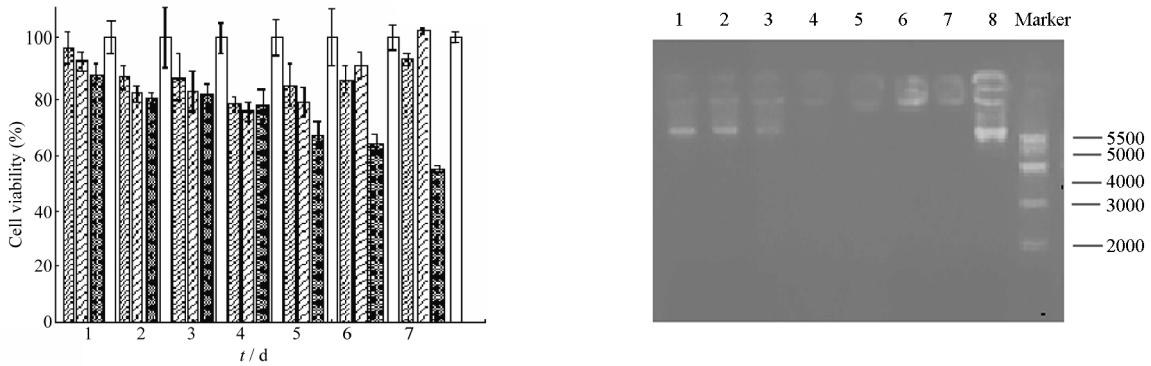
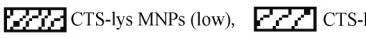


Fig.5 (A) Cell cytotoxicity of CTS-lys-MNPs and MNPs with U293 cells; (B) agarose gel electrophoresis retardation of DNA (pGFP) by CTS-lys-MNPs

Lane numbers correspond to different mass ratios of CTS-lys-MNPs/DNA as follows: (1) 1:5, (2) 1:4, (3) 1:2, (4) 1:1, (5) 2:1, (6) 4:1, (7) 5:1, (8) 0:1 (DNA only)



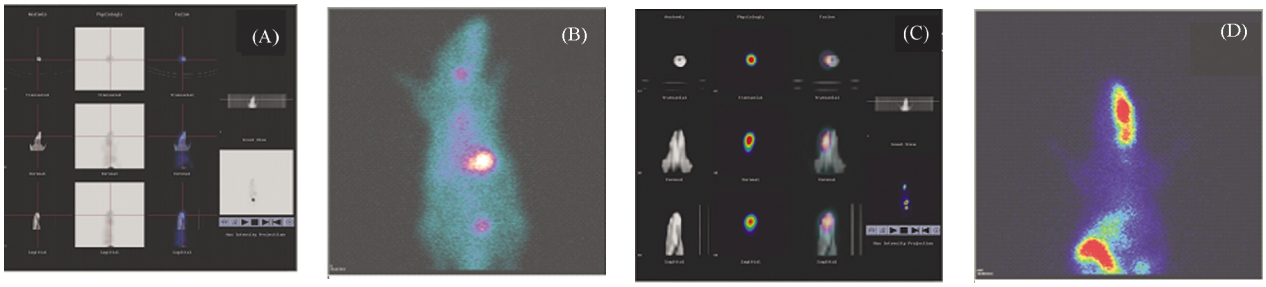


Fig.6 (A) SPECT of $^{99m}TcO_4^-$ in the brain of rat; (B) SPECT of $^{99m}TcO_4^-$ distribution in the body of rat; (C) SPECT of CTS-lys-MNPs/DNA complexes in the brain of rat; (D) SPECT of CTS-lys-MNPs/DNA complexes distribution in the body of rat

2.5 Ability of crossing BBB for CTS-lys-MNPs/DNA complexes *in vivo*

Fig.6(A) is the SPECT of radionuclide $^{99m}TcO_4^-$ in the brain of rat. Fig.6(C) is the SPECT of CTS-lys-MNPs/DNA complexes in the brain of rat. The main focus of this study is to examine whether the CTS-lys-MNPs/DNA complexes can pass the blood-brain barrier (BBB) *in vivo*[27]. From Fig.6(A), it can be seen that the radionuclide technetium^{-99m} cannot pass the BBB. After it is connected with CTS-lys-MNPs/DNA complexes, it is found that there are radiotracers in the rats′ brains (Fig.6(C)). Fig.6(B) shows that there is no obvious aggregation of radiotracers in the rats′ brains, but the image of the distribution of CTS-lys-MNPs/DNA complexes in the body of rat (Fig.6(D)) demonstrates that significantly high number complexes can be transported to the brain through the magnetic field. All these illustrate that the CTS-lys-MNPs/DNA complexes can pass the BBB under the magnetic field. And all the results illuminate the fact that CTS-lys-MNPs will be a new, safe gene delivery system.

3 Conclusions

We originally used the biocompatible, biodegradable, and nontoxic CTS-lys as a novel polymer matrix to prepare superparamagnetic nanospheres. After studying the influence of various parameters, We got the best recipe to prepare CTS-lys, where pH was 5, temperature was 20 ℃, stirring rate was 1200 r·min^{-1}, and the molar ratio of O/W was 1:1.

We intended to test whether CTS-lys-MNPs could be versatile gene carriers. The characterizations indicated that the average size of CTS-lys-MNPs was about 100 nm, and they had narrower size distribution, good superparamagnetic property, and perfect crystallinity. The result of the cytotoxicity showed that the CTS-lys-MNPs maintained low toxicity. Additionally, the combination of DNA and MNPs, which was observed from agarose gel electrophoresis, suggested that CTS-lys-MNPs could be novel magnetic targeting gene carriers.

In the *in vivo* study, we found that the CTS-lys-MNPs were successfully located in the brain of rats. The CTS-lys-MNPs successfully passed the BBB under the magnetic field. Therefore, the CTS-lys-MNPs are promising in therapy of tumor in brain such as glioma. We also have latent application in therapy of other diseases in the brain. We hypothesized that the magnetic targeting characteristic could be a superior method of carrying genes to the brain, and we also believed that this exciting technology might offer a creative advance in gene delivery.

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