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Fabrication IL-1Ra loaded galactosylated chitosan nanoparticles for liver targeting

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Abstract: Galactosylated chitosan (GC) is synthesized and used to prepare IL-1Ra loaded GC nanoparticles by an electrospraying technique. Polyethylene oxide (PEO) is mixed with GC to enhance the electrospraying ability. The effect of the spraying solution properties on particle formation is investigated. The IL-1Ra loaded nanoparticles with an average diameter of 530 nm and a regularly spherical shape are observed by the scanning electron microscopy (SEM). The amount of the IL-1Ra is measured by the enzyme-linked immunosorbent assay (ELISA) kit. The loading capacity of the nanoparticle is $(1.52 \pm 0.04)\%$ ($n = 3$) and the encapsulation efficiency reaches $(90.36 \pm 3.46)\%$ ($n = 3$). For the evaluation of GC nanoparticles' hepatocytes targeting efficacy, hepatocytes and mesenchymal stem cells (MSCs) are incubated with FITC-labeled GC nanoparticles for 24 h as the experimental and control groups. Results of the fluorescence microscope show that the fluorescence signals observed in hepatocytes are significantly higher than in the MSCs, indicating that the developed GC nanoparticles have an obvious liver targeting property.

Key words: nanoparticle; galactosylated chitosan; electrospraying; liver targeting
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Large numbers of protein drugs are being developed due to the significant advances in biotechnology[1]. However, an appropriate delivery approach is an important problem to face because of the relatively short circulating half-life in the body and the fragile nature of these therapeutic agents[2]. Protein drug-loaded biodegradable polymer nanoparticles which can control release and protect the non-released protein from degradation have become the most promising approach for the delivery of proteins[3-4]. The electrospraying technique, a process of utilizing a strong electric field to atomize liquid into charged droplets, has been reported as an innovative method in fabricating drug delivery particles[5]. A series of studies have shown that electrospraying is a "soft" enough method to formulate delivery carriers such as thin films and microcapsules and thus suitable for encapsulating biomacromolecules such as proteins, enzymes and DNA plasmids[6-7]. However, few studies have been reported on protein drug-loaded biodegradable polymer nanoparticles developed by electrospraying method so far.

Because of the donor organ shortage in liver transplantation therapy, cell transplantation is considered a promising alternative to treatment of hepatic failure. MSCs with the abilities to differentiate into functional hepatocytes have been reported[8]. However, the activity of the transplanted MSCs deteriorates with time due to the hepatic failureinflammatory milieu. In this study, IL-1Ra is selected as the model drug to pretreat the inflammation, and the aim is to improve the cell therapy efficiency.

Recently, the drug delivery carrier with site-specific properties has attracted extensive attention. Asialoglycoportein receptors (ASGP-R) which are expressed plentifully on the surfaces of hepatocytes can specifically recognize galactose and N-acetylgalactosamine-terminated glycoproteins[9]. Therefore, hepatocyte-specific delivery can be achieved by introducing galactose ligands into delivery carriers.

Chitosan (CS) is a natural biomaterial and exhibits excellent characteristic in biodegeneration, biocompatibility and non-immunoreaction[10]. GC is a derivative modified CS with galactose ligands. In previous studies, GC has been found to be a good hepatocyte-specific drug carrier[11]. In the electrospraying process, biomacromolecules' conformations may be changed due to the organic solvent environment. However, to the best of our knowledge, nanoparticles developed by the electrospraying technique and applied as a protein drug delivery carrier without an organic solvent has not been reported so far.

In this paper, we report a kind of IL-1Ra loaded GC nanoparticles which have an ability to reduce inflammation in hepatic failure disease. GC is synthesized by introducing LA into the CS, and then mixed with PEO to enhance the electrospraying ability via organic solvent-free electrospraying[12]. The size and morphology of the prepared nanoparticles are investigated by the SEM, and the

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hepatocyte targeting efficacy of the prepared nanoparticles is evaluated in vitro.

1 Materials and Methods

1.1 Materials

Chitosan (viscosity: 100 mPa · s, deacetylation: 85%) is purchased from TCI (Tokyo, Japan). Lactobionic acid (LA) is purchased from Acros Orgnics (Belgium). N-hydroxysuccinimide (NHS) and 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) are supplied by De-Bao Biochemistry (Nanjing, China). N, N, N, N-tetramethylethylenediamine (TEMED) is obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Poly (ethylene oxide) (MW: 10^6) is obtained from Guoren Chemical Co. (Beijing, China). All other reagents are of analytical reagent grade.

1.2 Synthesis of GC

CS is coupled with LA via an active ester using EDC and NHS as previously reported[13]. Briefly, 0.5 g of chitosan is dissolved in 50 mL of a TEMED/HCl buffer solution (10 mmol/L, pH = 4.7). Subsequently, 2.3 g of LA is activated with a mixture of NHS and EDC dissolved in 10 mL of TEMED/HCl buffer solution. EDC has a 4-fold molar excess over LA and EDC/NHS is at an equivalent molar ratio. Then, the activated LA is added into the CS solution and stirred for 72 h at room temperature. The resulting solution is purified and dialyzed using dialysis tube against distilled water for 4 d. GC is obtained by freeze drying at −50 ℃.

The infrared spectra of products are measured using the IR spectrometer (Bruker Vector22, Germany). Dried samples are grounded with KBr powder and compressed into disks for IR examination.

1.3 Eletrospraying of drug-loaded GC nanoparticles

The electrospraying setup is shown in Fig. 1. GC nanoparticles are prepared by electrospraying of a GC/PEO blend mixture. GC solutions are prepared by dissolving 100 mg of GC powders in the 0.5% acetic acid. Subsequently, PEO is added into the above solution with varying ratios. IL-1Ra is diluted in the PBS solution and then a specified amount of IL-1Ra is dropped into the above polymer mixture solutions and stirred for several hours. Each polymer/IL-1Ra solution fed by electrospraying is added into a 20 mL syringe with a 7-guage needle and placed onto a syringe pump (Smith, Zhejiang). A voltage power source (Dongwen, Tianjin) in the range of 0 to 40 kV is applied between the needle and a sheet of aluminium foil for collection. A stable Taylor cone is obtained at a flow rate of 0.4 mL/h, a working distance of 10 cm and a voltage of 14 kV. The obtained particles are stored in the dessicator at room temperature.

FITC-labeled GC nanoparticles are prepared for the hepatocyte targeting study. 3 mg of FITC is dissolved in the ethanol and then stirred with the GC/PEO solution for 3 h, and the following experimental procedure is the same as that of the GC nanoparticles described above. The synthesis of FITC-labeled GC is based on the reaction between the isothiocyanate group of FITC and the primary amino groups of GC[14].

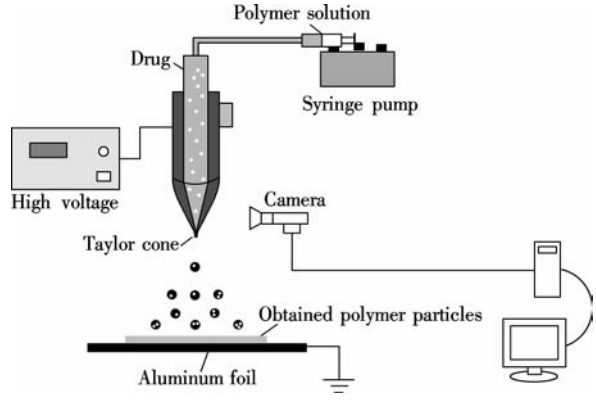

Fig. 1 Schematic illustration of the electrospraying setup used for particle preparation

Morphologies of the particles are observed with the SEM (Hitachi S-4800, Japan). The dry particle sample is sputter-coated with gold (30 s) before investigation. The mean diameter of electrosprayed nanoparticles is determined by image analysis of the SEM pictures. One hundred particles are selected randomly from the image to obtain the average diameter.

The bioactivity of the IL-1Ra and the number of the encapsulated IL-1Ra are measured by the ELISA method with an IL-1Ra assay kit (R&D, USA). The IL-1Ra loading capacity L_c and the encapsulation efficiency E_e are calculated by

$$L_c = \frac{W_1}{W_2} \times 100\% \ , \ E_e = \frac{W_1}{W_3} \times 100\%$$

where W_1 is the weight of drugs in the nanoparticles; W_2 is the total weight of nanoparticles; W_3 is the total weight of IL-1Ra added in the spraying solutions for the preparation of IL-1Ra loaded nanoparticles.

1.4 Cell isolation and culture

Hepatocytes are isolated from the livers of porcines by the two-step in situ collagenase perfusion technique[15]. Briefly, the portal vein and the inferior vena cava in porcine are cannulated. Hepatoctes are isolated by an in situ D-hanks solution and collagenase buffer perfusion. The perfused porcine liver is dissected and suspended in the PBS solution, and filtered through a 100 μm sterile mesh to remove tissue debris. Then the dead hepatocytes are removed by centrifugation (1 000 r/min, 5 min) at 4 ℃ and the viable hepatocytes suspension is determined by the Trypan blue exclusion test. Cells are seeded with an RPMI-1640 medium and incubated with 5% CO_2 at 37 ℃. Yields of the cell are maintained and calculated by

an inverted microscope.

1.5 Hepatocyte targeting

In this study, the hepatocyte is selected as the experimental group. Fresh primary hepatocytes (5×10^6 cells) are seeded onto sterile glass dishes in a 6-well microplate and cultured in RPMI-1640 medium with 5% of FBS, RPMR 1640, 100 IU/mL penicillin and 100 μg/mL streptomycin. The mesenchymal stem cells (MSCs) are used to contrast with the hepatocytes and serve as the control group. MSCs (5×10^6 cells) are also seeded onto sterile glass dishes in a 6-well microplate and cultured in DMEM medium containing 10% of FBS, DMEM, 100 IU/mL penicillin and 100 μg/mL streptomycin. After 24 h incubation at 37 ℃, the unattached cells are washed by PBS three times. 2 mL of FITC-labeled GC nanoparticles which are dispersed by PBS are added to both the groups and then 2 mL of RPMR1640 and DMEM medium are added to the cells, respectively. After being cultured for 24 h, the fluorescence images are obtained by the fluorescence microscope.

2 Results and Discussion

2.1 Synthesis of GC

The scheme used in the GC synthesis is shown in Fig. 2. In this reaction, the amide bond is obtained from coupling amine groups of CS and the carboxylic of LA with EDC and NHS as active esters[16]. Fig. 3 shows the IR spectra of CS, LA and GC. It can be seen that the carboxylic stretching (C═O) of LA at 1 745 cm^{-1} disappeares in GC spectrum. When comparing the spectra of GC with CS, it is found that the bands of amides Ⅰ and

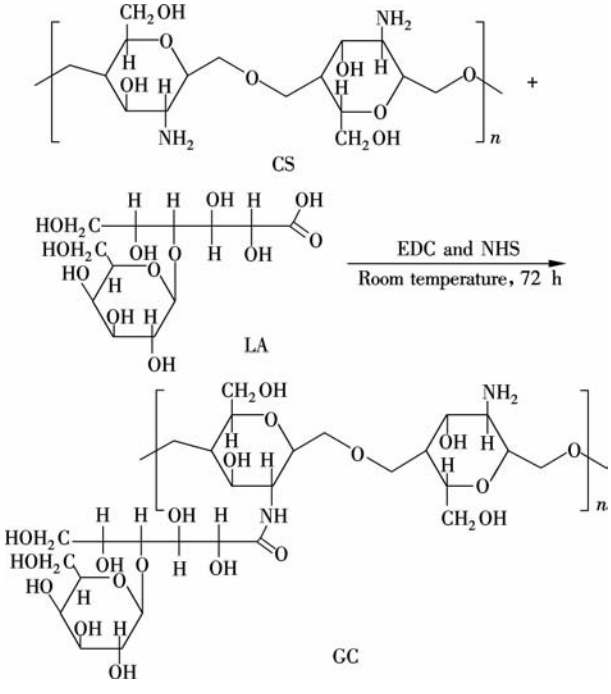

Fig. 2 Synthesis scheme of GC

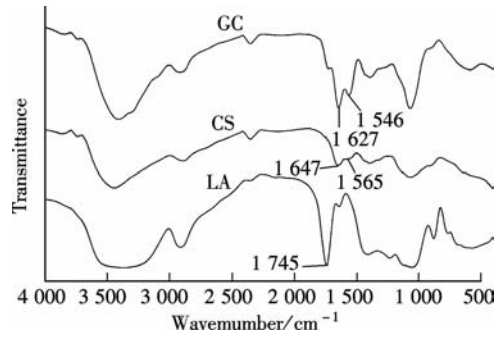

Fig. 3 IR spectra of GC, CS and LA

Ⅱ slightly shift from 1 647 and 1 565 cm^{-1} in the CS spectrum to 1 627 and 1 546 cm^{-1} in the GC spectrum, respectively. The conformation change of CS after reaction with LA indicates that the LA is introduced into the CS.

2.2 Characterization of nanoparticles

Electrospraying has been reported as a promising technique for fabricatemicro/nanoparticles. The fabrication parameters and spraying solution properties play a critical role in the morphology and size of particles[17]. The parameters such as the electrospray voltage, the flow rate and the working distance are optimized as described above. Tab. 1 lists the physical properties of the spraying solution including surface tension, viscosity and conductivity. It is observed that the viscosity of the solution increases as the PEO concentration increases. However, the surface tension and conductivity of the spraying solutions are observed small differences with the increase in PEO concentration. In addition, a pure GC aqueous solution with a concentration equal to or less than 1.7 kg/L cannot create a stable Taylor cone for electrospraying. PEO is introduced to GC aiming at obtaining a stable cone-jet via changing inter and intramolecular interactions of GC chains. Fig. 4 shows the SEM images of particles obtained from different GC/PEO concentration ratios. When the concentration of the PEO increases from 0.15 to 0.75 kg/L (S2, S3 and S4) with constant IL-1Ra/GC, the surface morphologies of the particles are changed from spherical shapes to fibrous. So we choose S3 as the optimized electrospraying solution parameters to fabricate nanoparticles. Fig. 5 shows the average diameter and size distribution of the obtained nanoparticles, and the average diameter of the prepared nanoparticles is 530 nm.

The bioactivity test shows that about 90% of the bioactivity of IL-1Ra is maintained by the electrospraying method. The IL-1Ra loading capacity and encapsulation efficiency of the nanoparticles are (1.52 ± 0.04) % (n = 3) and (90.36 ± 3.46) % (n = 3), respectively. It indicates that the electrospraying method can produce protein loaded nanoparticles with good encapsulation efficiency.

Tab. 1 Characteristics of spraying solutions prepared

Solution	C_{GC}/(kg · L^{-1})	C_{PEO}/(kg · L^{-1})	IL-1Ra/mg	Surface tension/(mN · m^{-1})	Viscosity/(mPa · s)	Conductivity/(S · cm^{-1})
S1	1.7	0	1.7	57.0	328.0	186.9
S2	1.3	0.15	1.7	58.0	234.0	185.3
S3	1.3	0.35	1.7	59.0	268.0	189.0
S4	1.3	0.75	1.7	55.9	370.0	185.5

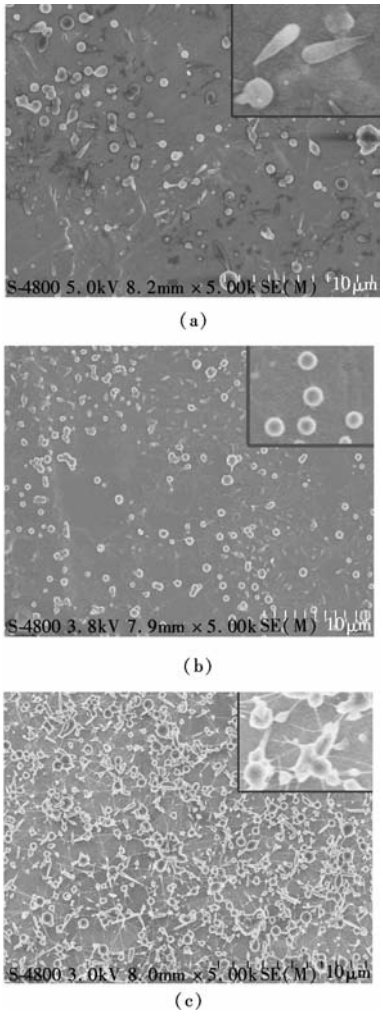

Fig. 4 SEM images of IL-1Ra loaded nanoparticles electrosprayed from 0.5% acetic acid solutions containing. (a) 1.3 kg/L GC and 0.15 kg/L PEO; (b) 1.3 kg/L GC and 0.35 kg/L PEO; (c) 1.3 kg/L GC and 0.75 kg/L PEO

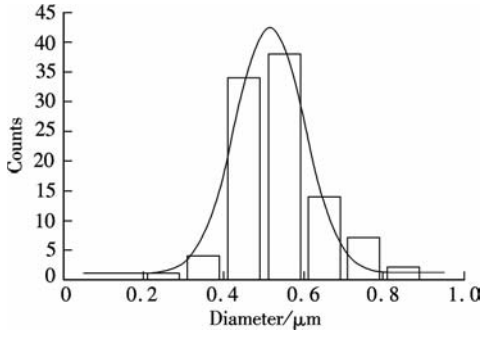

Fig. 5 Diameter distribution of particles electrosprayed from 0.5% acetic acid solutions containing(1.3 kg/L GC and 0.35 kg/L PEO)

2.3 Images of cells

Fig. 6 shows the image of fresh hepatocytes observed from the inverted microscope. It can be seen that the fresh hepaptocytes are translucent and round. Figs. 7(a) and (b) show the results of the fluorescence images of hepatocytes and MSCs incubation with the FITC-labeled GC nanoparticles for 24 h. The fluorescence signal observed in the MSCs group is not significant. In contrast, due to the targeting ability of the galactose residues of the LA introduced into the CS, the fluorescence signals observed in hepatocytes are obviously higher than those of MSCs. The results of the fluorescence images suggest that because of the specific interactions between the ASGP receptor and galactose residues, the GC nanoparticle carriers have the hepatocyte-specific properties.

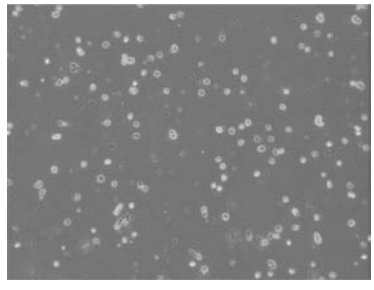

Fig. 6 Image of the fresh hepatocytes observed from inverted microscope

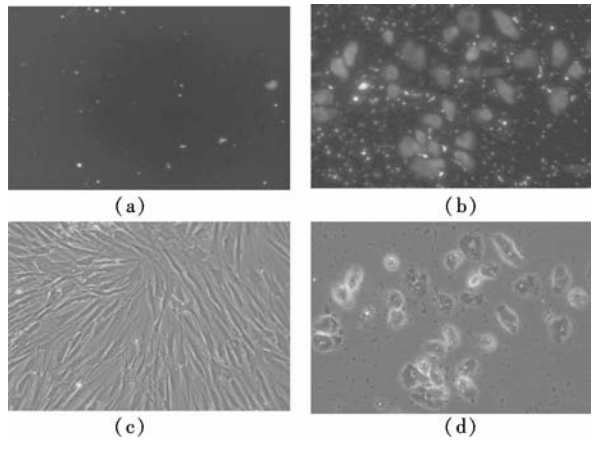

Fig. 7 Fluorescence images of hepatocytes and MSCs incubated with the FITC-labeled GC nanoparticles for 24 h. (a) MSCs observed in fluorescence; (b) Hepatocytes observed in fluorescence; (c) MSCs observed in visible light; (d) Hepatocytes observed in visible light

3 Conclusion

In this study, we develop a GC-based nanoparticles

with uniform size distribution of 530 nm by organic solvent-free electrospraying in the Taylor cone-jet mode. IL-1Ra is successfully encapsulated in the nanoparticles with smooth surfaces. The results of the study indicate that the nanoparticles conjugated with galactose residues have a high affinity to hepatocytes. It is concluded that the prepared GC nanoparticles are good drug carriers for the liver-targeting delivery and the electrospraying method is a promising approach for encapsulating bioactive materials.

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肝靶向白介素 1 受体拮抗剂乳糖酰基壳聚糖纳米颗粒的制备

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摘要:合成乳糖酰基壳聚糖(GC)并以此为载体材料,采用静电喷射法制备载白介素 1 受体拮抗剂(IL-1Ra)GC 纳米颗粒.在实验过程中,加入一定量聚氧化乙烯(PEO)于 GC 电纺溶液中,考察电纺溶液性质对颗粒形成的影响,优化溶液性质参数,通过扫描电子显微镜(SEM)观察得到表面光滑、颗粒平均直径约为 530 nm 的载 IL-1Ra 纳米颗粒,并通过酶联免疫(ELISA)试剂盒检测得到载药纳米颗粒包封率为(1.52 ± 0.04)%(n = 3),载药率达到(90.36 ± 3.46)%(n = 3).为了考察所制备的 GC 纳米颗粒对肝细胞的亲和靶向性,实验以猪肝细胞为实验组,猪骨髓基质干细胞为实验对照组,分别加入 FITC 标记的 GC 纳米颗粒培养 24 h,荧光显微镜下观察到猪肝细胞表面荧光信号明显强于骨髓基质干细胞,表明本实验所制备的 GC 纳米颗粒具有明显的肝靶向功能.

关键词:纳米颗粒;乳糖酰基壳聚糖;静电喷射;肝靶向

中图分类号:TQ31