ISSN : 0974 - 7494 Volume 5 Issue 1

Trade Science Inc.

Nano Science and Nano Technology
An Indian Journal

Full Paper

NSNTAIJ, 5(1), 2011 [44-50]

Preparation and characterization of aqueous carboxymethyl chitosan-coated ferrofluid as DNA carrier

W.Zhang[1]*, Z.L.Yuan[1], Z.F.Hu[1], L.Zhuang[2], X.P.Zhang[3], H.Y.He[1]

[1]School of Physics and Optoelectronic Engineering, Guangdong University of Technology, Guangzhou 510006, (PEOPLE'S REPUBLIC OF CHINA)

[2]Institute for Solar Energy Systems, State Key Laboratory of Optoelectronic Materials and Technologies, Sun Yat-Sen University, Guangzhou, 510275, (PEOPLE'S REPUBLIC OF CHINA)

[3]Cancer Hospital of Guangzhou Medical College, Guangzhou, 510095, (PEOPLE'S REPUBLIC OF CHINA)

Received: 28th October, 2010 ; Accepted: 7th November, 2010

ABSTRACT

A one-step method of preparing Fe_3O_4 magnetic nanoparticles coated with carboxymethyl chitosan was evaluated. The coated magnetic nanoparticles showed high saturate magnetism strength (σ_s = 37.5 emμ/g). The diameter of modified nanoparticles was approximately 14.5 nm. Furthermore, the binding between modified magnetic nanoparticles and DNA was examined by agarose gel electrophoresis. The preliminary results demonstrated that carboxymethyl-chitosan-modified Fe_3O_4 magnetic nanoparticles might be a potential DNA carrier.

KEYWORDS

Fe_3O_4;
Nanoparticle;
Carboxymethyl chitosan;
DNA carrier.

INTRODUCTION

In the recent years, the biocompatible aqueous-based magnetic nanoparticles receive increasing attention along with rapid development of nano-structured materials and nanotechnology[1,2]. Aqueous-based magnetic fluids might find applications in magnetic fluids-mediated intracellular hyperthermia, magnetic resonance imaging and magnetic targeting drug delivery[3-6]. All these biomedical applications require that these magnetic nanoparticles have long-term stability in aqueous solutions, smaller size (< 100 nm), and high magnetization values. One approach for the preparation of aqueous-based magnetic fluids is to coat the magnetite nanoparticles with surfactant bilayers[7,8]. Chitosan, a polysaccharide composed of D-glucosamine and N-acetyl-D-glucosamine, has been used to modify the magnetite nanoparticles[9,10]. Since the amino groups can serve as chelation sites, chitosan and its derivatives are capable of adsorbing DNA molecules and utilized as a DNA carrier[11,12].

Carboxymethyl chitosan (Cmch) has great potential applications in biotechnology, biomedicine, food ingredients, and cosmetics[13,14]. Note worthily, carboxymethyl chitosan is also capable of binding drugs because it has amino and carboxyl groups which can serve as chelation sites. Thus, the binding of carboxymethyl chitosan with aqueous-based magnetic fluids will probably yield another novel magnetic nano-adsorbent as a DNA carrier.

The preparations of carboxymethyl chitosan-coated aqueous-based magnetic fluids by co-precipitation,

crosslink, and covalent binding using coupling agents have been reported[15]. The preparation and optimization of carboxymethyl chitosan -conjugated aqueous-based magnetic fluids certainly need further study. Since carboxymethyl chitosan has suitable functional groups such as carboxyl groups, they can bind directly onto Fe_3O_4 nanoparticles and be dispersed in water. In this study, carboxymethyl chitosan-coated magnetic fluids were studied. The capability of DNA binding was also investigated.

MATERIALS AND METHODS

Chemicals

Carboxymethyl chitosan (molecular weight = 20 kDa, 80% substitution) were purchased from Heppe Biotechnology Company (Qingdao, China). Plasma DNA (PUC18, 150 μg/ml) was obtained from Takara Company (Shiga, Japan). All other chemicals (AR) were obtained from Guangzhou Chemical Company and Guangzhou Whiga Technology Company.

Preparation of carboxymethyl chitosan coated magnetic nanoparticles

Fe^{2+}-Fe^{3+} mixture (1:1.5-2) was prepared by mixing 21.9% ferrous chloride ($FeCl_2.4H_2O$) and 66.6% ferric chlorides ($FeCl_3.6H_2O$). Fresh carboxymethyl chitosan solution was prepared by dissolving 2 g of carboxymethyl chitosan powder with 30 ml of ammonia solution ($NH_3.H_2O$, 25 wt. %).

To coat the magnetic nanoparticles, 9 ml of the Fe^{2+}-Fe^{3+} solution was added to a flask containing 30 ml of carboxymethyl chitosan solution under high speed stirring at 90 °C. First, the mixture was stirred at 1200 r/min for 10 min and then at 625 r/min for 50 min. Fe^{2+}-Fe^{3+}-chitosan polymers were centrifuged at 1000 r/min for 4 h at room temperature to remove large particles. The magnetic supernatant was mixed with 24 ml of distilled water and subjected to ultrafiltration to remove surplus carboxymethyl chitosan. The ultrafiltration was carried out in a SCM cup-type ultra-filter (Shanghai Yadong Hitech Company) using an ultrafiltration film (molecular weight of 30 kDa; PS–30, Shanghai Yadong Hitech Company) under nitrogen gas purging. Filtration procedure was repeated for 6 times using 12 ml of distilled water. Fe^{2+}-Fe^{3+}-chitosan polymers (Cmch-Fe_3O_4) collected on the filter was dispersed into 10 ml distilled water by ultrasound and stored at 4°C.

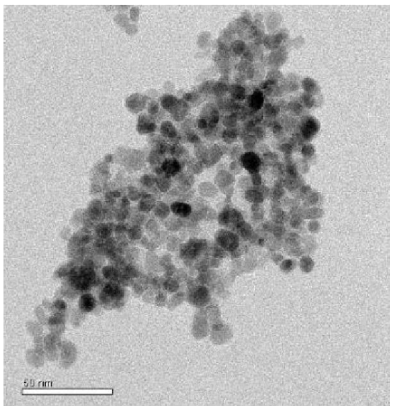


Figure 1 : The TEM micrograph of nanoparticles of Cmch-Fe_3O_4

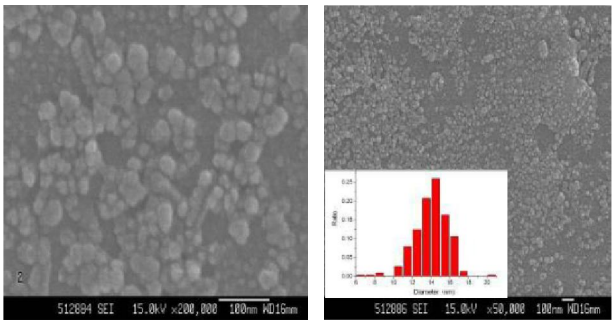


Figure 2 : The SEM nanoparticles of Cmch-Fe_3O_4. (insert: diameters distribution)

Characterization of chitosan modified magnetic nanoparticles

(a) Electron microscope analyses

Transmission electron microscope (TEM) micrographs of coated Fe_3O_4 particles were taken on a high-resolution electronic microscope (JEM-2010HR, JEOL) with an accelerating voltage of 80 kV. The average particle size was obtained by measuring numerous particles of similar size from several images. The scanning electron microphotographs (SEM) of coated Fe_3O_4 particles were obtained from a field emission scanning microscope (JSM-6330F, JEOL). The average particle size was obtained by measuring numerous particles of similar size from several micrographs.

(b) Fourier transform infrared spectra analysis

Fourier transform infrared (FT-IR) spectra of uncoated and coated Fe_3O_4 particles were recorded on a FT-IR spectrometer (Bio-Rad FTS 3000). After adjusting the pH value to 6.0, samples were dried at

Full Paper

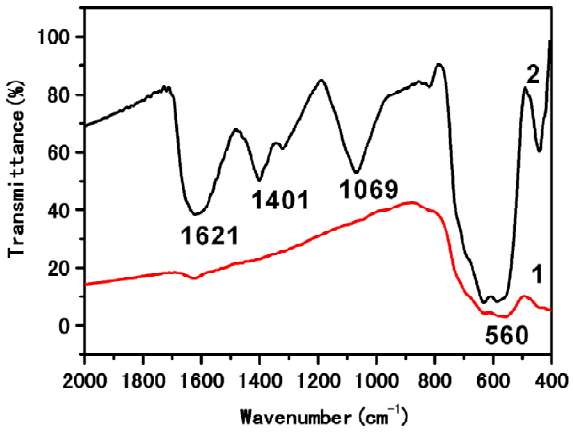


Figure 3 : IR spectrum. Uncoated Fe_3O_4 nanoparticles (line 1) and Cmch-Fe_3O_4 (line 2)

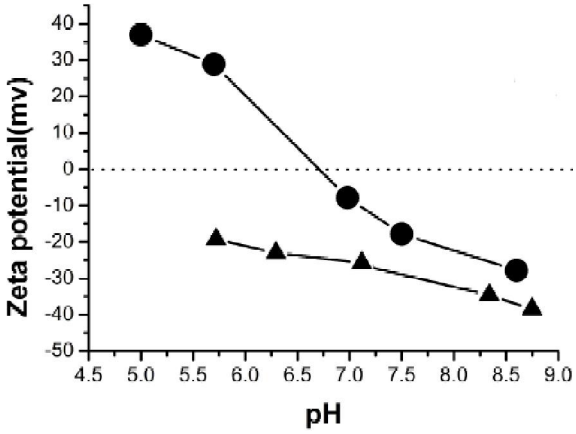


Figure 4 : Zeta potentials curve of Uncoated Fe_3O_4 (•) and Cmch-Fe_3O_4 (▲) as a function of pH

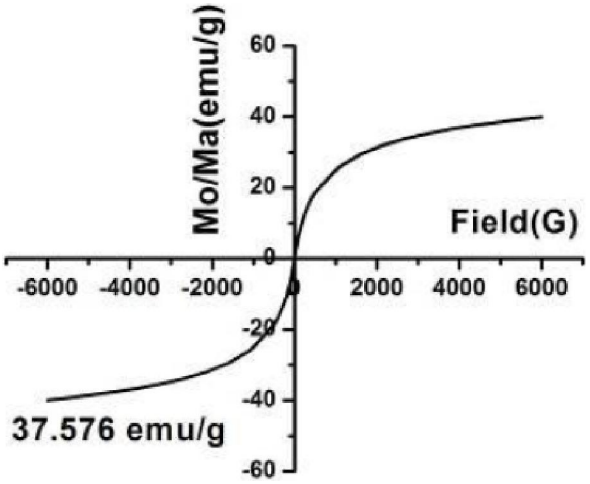
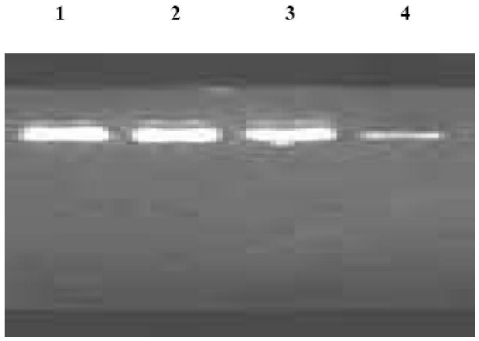


Figure 5 : The magnetization curve of nanoparticles of Cmch-Fe_3O_4



Lane 1: 6 µl of distilled water + 3 µl of DNA (300 µg/ml),
Lane 2: 4 µl of Cmch (2 mg/ml) + 3 µl of DNA + 4 µl of buffer (pH = 6.83),
Lane 3: 4 µl of Cmch(3 mg/ml) + 3 µl of DNA + 4 µl of buffer,
Lane 4: 4 µl of Cmch-Fe_3O_4 (1 mg/ml) + 3 µl of DNA + 4 µl of buffer (DNA:Fe^{3+} = 9:40)

Figure 6 : Binding between carboxymethyl chitosan coated magnetic liquid and DNA.

80°C in vacuum, mixed with KBr powder and then pressed to thin pellets (~ 0.055 mm thickness) for IR examinations.

(c) Zeta potential analysis

Zeta potentials of uncoated and coated Fe_3O_4 were measured by a Coulter Delsa 440SX zeta potential analyzer (Beckman-Coulter) at 25°C. The pH of the suspensions (0.01 mg Fe/ml) were adjusted to 5-8.65 (uncoated) and 5.75-8.75 (coated) by addition of HCl (0.1 mol/l) or $NH_3.H_2O$ (0.1 mol/l). Measurement was repeated 2-3 times for each pH point.

(d) Magnetization analysis

The magnetization curve of coated Fe_3O_4 fluids was measured on a magnetometer (Lakeshore 7404 vibration) at 25°C.

DNA binding on chitosan modified magnetic nanoparticles

Three µl of plasmid DNA (300 µg/ml) was mixed with 3 µl of Cmch-Fe_3O_4 (1.6 mg/ml, filtered) and 4 µl of phosphate buffer (pH = 6.83) and incubated at 4°C for 90 min. Ten µl of mixture (0.5-7 mg/ml based on Fe' ionic concentration) were loaded on 1% agarose gel and electrophoresed in TAE buffer at 100 V for 15-20 min. DNA was visualized with 1% ethidium bromide. To examine the effect of pH on the DNA binding, the above procedure was repeated by incubating Cmch-Fe_3O_4-DNA in phosphate buffer of different pH (4.56, 6.83 and 8.98) or distilled water. To examine the effect of filtration, un-filtered Cmch-Fe_3O_4-DNA mixture was diluted (1:5) in Tris-Acetate-EDTA (TAE) buffer and incubated for 90 min before being subjected to gel electrophoresis.

RESULTS

Characterization of chitosan modified magnetic nanoparticle

TEM and SEM examinations showed that the morphology of carboxymethyl chitosan -coated Fe_3O_4 particles were spherical shape (Figure 1 and 2). Most particles were well dispersed except a small portion of aggregation, possibly formed during sample preparation. Since the carboxymethyl chitosan layers were not a crystal and therefore were invisible under TEM, the particle size seen under the TEM should represent the size of Fe_3O_4 crystal core. The average diameter measured under TEM was 8 nm. SEM images showed that all the particles were carboxymethyl chitosan coated, which formed a continuous and dense coating layer with a narrow distribution of particle size around 15 nm and chitosan shell thickness of 3-4 nm (Figure 2).

IR spectra of uncoated and coated Fe_3O_4 nanoparticles are shown in figure 3. The two main characteristic peaks of line 2 at 1621 and 1401 cm^{-1} represented the bands of -COOM groups (M = mental ions), which indicated that the -COOH groups reacted with the surface -OH groups of Fe_3O_4 particles and resulted in the formation of the iron carboxylate[16-18]. The peak at 1069 cm^{-1} was the characteristic band of carboxymethyl chitosan and that at 560 cm^{-1} was the characteristic band of uncoated-Fe_3O_4. These results indicated that monolayer carboxymethyl chitosan molecules had been chemisorbed on the nanoparticles' surfaces.

The zeta potentials were positive at pH above the isoelectric point and were negative below the isoelectric point[19]. The isoelectric point of uncoated Fe_3O_4 nanoparticles was around pH = 6.5. As shown in the figure 4, the zeta potentials of Cmch-coated Fe_3O_4 nanoparticles decreased from pH 5.5 to 9 and the isoelectric points of Cmch-coated Fe_3O_4 nanoparticles shifted towards lower pH values. At pH = 6.5, the Cmch-bound ferrofluid had a negative zeta potential, which confirmed that Fe_3O_4 nanoparticles were coated with Cmch. At a certain pH range (e.g. 5.5 to 9), the surface charge of nanoparticles depended on the $-NH_3^+$ groups and the free $-COO^-$ groups adsorbed on the Cmch chains. Simply, the surface charge was proportional to the net charges of $-NH_3^+$ and $-COO^-$ groups. Therefore, the surface charge of nanoparticles actually depended on the degree of carboxylation of chitosan (i.e. the amount of $-NH_2$ groups replaced by -COOH groups at the surface of chitosan). The higher the degree of carboxylation, the more negative the surface potentials of Cmch-bound ferrofluid were.

The typical magnetization curve of a concentrated magnetic Fe_3O_4 nanoparticles stabilized by carboxymethyl chitosan is depicted in figure 5. It exhibited a good super-paramagnetic property with zero coercively and remanence and its saturation magnetization was about 37.5 emu/g[20]. The weak hysteresis revealed that the resultant magnetic nanoparticles were nearly superparamagnetic.

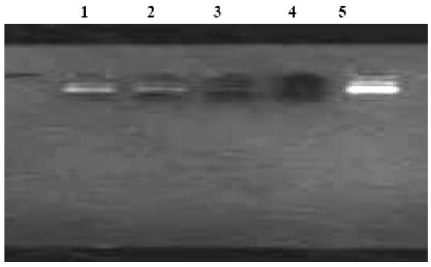
DNA binding on carboxymethyl chitosan modified magnetic nanoparticles

DNA binding on the carboxymethyl chitosan-Fe_3O_4 nanoparticles was visualized with ethidium bromide on agarose gel[21]. The bright band after electrophoresis represented free DNA. At DNA to Fe^{3+} mass ratio of 9:40 (i.e. 3 µl of DNA (300 µg/ml) in 4 µl of carboxymethyl chitosan-Fe_3O_4 (1 mg/ml)), DNA was not entirely absorbed by carboxymethyl chitosan-Fe_3O_4 nanoparticles after 90 min incubation (Figure 6, Lane 4). However, at DNA to Fe'ionic ratio of 3:80 (i.e. 3 µl of DNA (300 µg/ml) in 4 µl of carboxymethyl chitosan-Fe_3O_4 (6 mg/ml), DNA could be entirely absorbed by carboxymethyl chitosan-Fe_3O_4 nanoparticles after 90 min incubation (Figure 7, Lane 4). The pH value (4.56–8.98) showed little effect on DNA binding (Figure 8). Similar results were also seen with the unfiltered carboxymethyl chitosan-Fe_3O_4 nanoparticles (Figure 9, Lane 4).

DISCUSSION

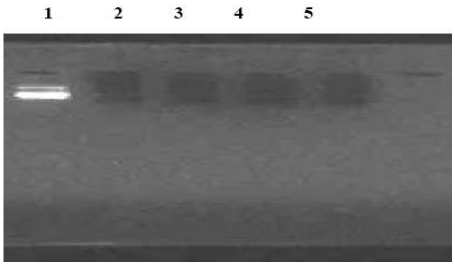
The preparation of carboxymethyl chitosan-coated aqueous-based magnetic fluids has been reported previously. It typically involves multiple steps such as coprecipitation, crosslink, and covalent binding using coupling agents[15]. Since carboxymethyl chitosan has suitable functional groups (e.g. carboxyl groups) which can bind directly onto Fe_3O_4 nanoparticles and be dispersed in water, based on this mechanism, we developed a one-step method to prepare carboxymethyl chitosan-

Full Paper



Lane 1: 4 µl of Cmch-Fe_3O_4 (1 mg/ml) + 3 µl of DNA (300 µg/ml) + 4 µl of buffer (pH = 6.83) (DNA:Fe^{3+} = 9:40),
Lane 2: 4 µl of Cmch-Fe_3O_4 (1.5 mg/ml) + 3 µl of DNA + 4 µl of buffer (DNA:Fe^{3+} = 3:20),
Lane 3: 4 µl of Cmch-Fe_3O_4 (3 mg/ml) + 3 µl of DNA + 4 µl of buffer (DNA;Fe^{3+} = 3:40),
Lane 4: 4 µl of Cmch-Fe_3O_4 (6 mg/ml) + 3 µl of DNA + 4 µl of buffer (DNA:Fe^{3+} = 3:80),
Lane 5: 6 µl of distilled water + 3 µl of DNA

Figure 7 : Effect of magnetic liquid concentration on binding between carboxymethyl chitosan coated magnetic liquid and DNA.



Lane 1: 6 µl of distilled water + 3 µl of DNA (300 µg/ml),
Lane 2: 4 µl of Cmch-Fe_3O_4 (6 mg/ml) + 3 µl of DNA + 4 µl of buffer (pH = 4.56),
Lane 3: 4 µl of Cmch-Fe_3O_4 (6 mg/ml) + 3 µl of DNA + 4 µl of buffer (pH = 6.83),
Lane 4: 4 µl of Cmch-Fe_3O_4 (6 mg/ml) + 3 µl of DNA + 4 µl of buffer (pH = 8.98),
Lane 5: 4 µl of Cmch-Fe_3O_4 (6 mg/ml) + 3 µl of DNA + 4 µl of distilled water (DNA:Fe^{3+} = 3:80)

Figure 8 : Effect of pH value on binding between carboxymethyl chitosan coated magnetic liquid and DNA.

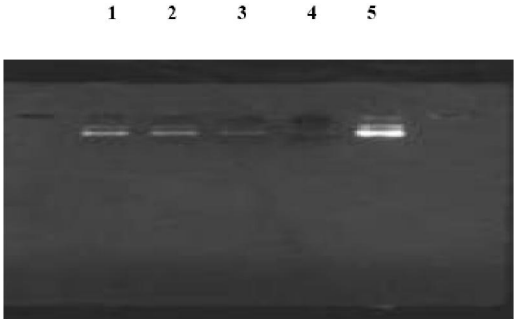
conjugated aqueous-based magnetic fluids.

TEM and SEM examinations showed that most particles were well dispersed and in spherical shape with smooth surface (Figure 1 and 2). SEM images showed that all the Fe_3O_4 particles were carboxymethyl chitosan coated, which formed a continuous and dense coating layer with a narrow distribution of particle size around 14.5 nm. The thickness of chitosan shell was estimated at 3-4 nm. This might be caused by the chain structure and stretching state of carboxymethyl chitosan molecules in water. Nonetheless, coated Fe_3O_4 nanoparticles were essentially monodispersed with similar mean diameter by compared with the nanoparticles prepared by two-steps co-precipitation method[19]. This reveals that the binding process did not significantly result in the agglomeration and more change in size of the particles. No change was observed for the actually thickness of adsorption layer.

IR absorption spectrum showed the adsorption peaks at 1621 and 1401 cm^{-1}, respectively (Figure 3). These peaks can be assigned to the bands of COOM groups (M = mental ions). This is in fair agreement with carboxymethyl being chemical binded to Fe^{3+} at the surfaces of Fe_3O_4 nanoparticles. This is also in agreement with recent theoretical simulations of formic, acetic and citric acids[23,24]. Therefore, it is reasonable to characterize that the adsorption of carboxymethyl chitosan to Fe_3O_4 nanoparticles surfaces is due to chemisorptions.

The zeta potentials of uncoated and coated Fe_3O_4 nanoparticles were different (Figure 4). At pH 6.5 (the isoelectric point of uncoated Fe_3O_4 nanoparticles), the Cmch-bound ferrofluid had a negative zeta potential, which confirmed that Fe_3O_4 nanoparticles were coated with Cmch and subsequently causing the surface charge change. The isoelectric point of uncoated Fe_3O_4 nanoparticles is at pH=6.5 or so[25]. The surface charge is negative pH=6.5 as carboxymethyl chitosan was hydrolyzed[19]. The electrical potential falls. The magnetization magnitude of uncoated samples was higher than that of particles bond with carboxymethyl chitosan. This is because the adsorption is strong or the quantity of carboxymethyl chitosan absorbed by Fe_3O_4 nanoparticles is higher and the amount of magnetic particles per unit quality is lower. As a result, the saturation magnetization of coated samples is lower.

Agarose gel electrophoresis was used to demonstrate the DNA binding on pure carboxymethyl chitosan and magnetic particles-modified with carboxymethyl chitosan. Our results revealed that DNA perhaps cannot bind to dissociated carboxymethyl chitosan and DNA can be connected to magnetic particles-modified with carboxymethyl chitosan (Figure 6). This might attribute to the surface potential. The surface potential of nanoparticles depends on the carboxylating degree of chitosan (i.e. amount of -NH_2 groups are replaced by -COOH groups at the surface of chitosan). Because the scale of the net charges of -COO^- groups and -NH_3^+ groups for pure carboxymethyl chitosan dispersed in water is higher than that of magnetic fluid, the surface



Lane 1: 4 µl of unfiltrated Cmch-Fe_3O_4 (1 mg/ml) + 3 µl of DNA + 4 µl of buffer (DNA:Fe^{3+} = 9:40),
Lane 2: 4 µl of unfiltrated Cmch-Fe_3O_4 (1.5 mg/ml) + 3 µl of DNA + 4 µl of buffer (DNA:Fe^{3+} = 3:20),
Lane 3: 4 µl of unfiltrated Cmch-Fe_3O_4 (3 mg/ml) + 3 µl of DNA + 4 µl of buffer (DNA:Fe^{3+} = 3:40),
Lane 4: 4 µl of unfiltrated Cmch-Fe_3O_4 (6 mg/ml) + 3 µl of DNA + 4 µl of buffer (DNA:Fe^{3+} = 3:80),
Lane 5: 6 µl of distilled water + 3 µl of DNA (300 µg/ml)

Figure 9 : Different concentration magnetic liquid of unfiltrated connection with DNA.

potential of carboxymethyl chitosan is higher at the same pH. Moreover, since the surface charge of DNA is negative, it is difficult to be bond to -NH_2 at the surface of carboxymethyl chitosan due to the electricity excluding force. In magnetic fluid there will be chemical reactions between -COO^- of carboxymethyl chitosan and Fe_3O_4, the surface potential of magnetic fluid is therefore lower than that of pure carboxymethyl chitosan. DNA can be bond to -NH_2 at the surface of carboxymethyl chitosan due to the reduction of the excluding force between magnetic particles and DNA. For future research, the low carboxylating degree of chitosan should be chosen in order to reduce the surface negative potential of magnetic fluid to facilitate DNA bond on magnetic particles.

The adsorption of DNA at different magnetic fluid concentration was investigated at pH 6.83, 25°C and a series of concentrations (1-6 mg/ml). As shown in figure 7, the light strip became brighter at lower concentrations. This might be attributed to the quantities of carboxymethyl chitosan that the binding DNA was lower at lower concentration. So DNA binding effect was better at a higher concentration. The effect of pH on the adsorption of DNA by the magnetic carboxymethyl chitosan nano-adsorbent at 25°C and an initial concentration of 6 mg/ml was illustrated in figure 8. At pH range of 4.5-9, the pH value had little influence on DNA binding. The adsorption of DNA at different concentrations of magnetic fluid with or without ultrafiltration was investigated. Results showed that the ultrafiltration was a critical step (Figure 9). These results were in agreement with that the pH value had little influence on DNA binding and the impurity ion (such as -NH_3^+) must be removed by ultrafiltration.

In summary, magnetic nanoparticles obtained by one-step method of chemical co-precipitation appeared to be spherical and dispersed well. The average particle size was 14.5 nm. Its saturation magnetization was 37.5 emµ/g. The analysis of agarose gel electrophoresis revealed DNA can be bound on to carboxymethyl chitosan-coated magnetic nanoparticles. The nanoparticles prepared were expected to be useful as a DNA carrier.

ACKNOWLEDGEMENT

This work was financially supported by the National Natural Science Foundation of China (Grant no. 30672297) and the Guangdong Provincial Natural Science Foundation of China (Grant no. 07300540).

REFERENCES

[1] D.Luo, W.M.Saltzman; Nat.Biotechnol., **18**, 33 **(2000)**.
[2] H.L.Jiang, Y.K.Kim, R.Arote, J.W.Nah, M.H.Cho, Y.J.Choi, T.Akaike, C.S.Cho; J.Control.Release, **117**, 273 **(2007)**.
[3] A.K.Guptaa, M.Gupta; Biomaterials, **26**, 3995 **(2005)**.
[4] S.W.Gersting, U.Schillinger, J.Lausiier, P.Nicklaus, C.Rudolph, C.Plank, D.Reinhardt, J.Rosenecker; J.Gene Med., **6**, 913 **(2004)**.
[5] J.M.Qu, G.Liu, Y.M.Wang, R.Y.Hong; Adv.Powder Technol., **21**, 461 **(2010)**.
[6] Y.Qiang, J.J.Antony, A.Sharma, J.Nutting, D.Sikes, D.Meyer; J.Nanopart Res., **8**, 489 **(2006)**.
[7] M.R.Saboktakin, A.Maharramov, M.A.Ramazanov; Carbohydr.Polym., **78**, 292 **(2009)**.
[8] Y.Guari, J.Larionova, K.Molvinger, B.Folch; Chem. Commun., **24**, 2613 **(2006)**.
[9] K.Turan, K.Nagata; Pharm.Dev.Technol., **11**, 503 **(2006)**.
[10] W.D.Cao, C.J.Easley, J.P.Ferrance, J.P.Landers; Ferrance, **78**, 7222 **(2006)**.
[11] J.Shao, Y.Yang; Acta Med.Nantong., **2**, 155 **(2000)**.

Full Paper

[12] C.Zhang, P.Yadava, J.Hughes; Methods, **33**, 144 **(2004)**.
[13] M.Safarýkova, I.Safarýk; Biotechnol.Lett., **22**, 941 **(2000)**.
[14] J.H.Park, K.H.Im, S.H.Lee, D.H.Kim, D.Y.Lee, Y.K.Lee, K.M.Kim, K.N.Kim; J.Magn.Magn. Mater., **293**, 328 **(2005)**.
[15] Y.C.Chang, S.W.Chang, D.H.Chen; React.Funct.Polym., **66**, 335 **(2006)**.
[16] Y.F.Zhou, T.Y.Fan, R.J.Yang, H.L.Sun, Q.S.Xia; Chinese J.Pharm.Sci., **16**, 187 **(2007)**.
[17] W.Zhang, H.Shen, J.H.Xia, X.S.He; Nanosci.Nanotechnol., **6**, 37 **(2006)**.
[18] S.Yasodhai, T.Sivakumar, S.Govindarajan; Thermochim.Acta, **338**, 57 **(1999)**.
[19] Y.C.Chang, D.H.Chen; J.Colloid.Interf.Sci., **2**, 446 **(2005)**.
[20] X.Q.Xu, H.Shen, J.R.Xu, M.Q.Xie, X.J.Li; Appl. Surf.Sci., **221**, 430 **(2004)**.
[21] C.L.Chiang, C.S.Sung; J.Magn.Magn.Mater., **302**, 7 **(2006)**.
[22] C.Brus, H.Petersen, A.Aigner, F.Czubayko, T.Kissel, T.Kissel; Eur.J.Phar.Biopharm., **57**, 427 **(2004)**.
[23] Y.H.Guo, F.R.Li, S.Y.Bao, T.Han, J.J.Cao, H.X.Zhou; Curr.Appl.Phys., **7S1**, e97 **(2007)**.
[24] L.Ojamae, C.Aulin, H.Pedersen, P.Kall; J.Colloid Interf.Sci., **296**, 71 **(2006)**.
[25] Y.Wu, J.Guo, W.L.Yang, C.C.Wang; Polym., **47**, 5287 **(2006)**.