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Self-assembled nanoparticle drug delivery systems from galactosylated polysaccharide–doxorubicin conjugate loaded doxorubicin

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ABSTRACT

Xyloglucan was grafted with the doxorubicin (DOX) and galactosamine, a terminal moiety that can be used to target polymeric conjugates to liver hepatocytes. The content of the DOX was over 5% (wt) in the conjugate. The polymeric drug assisted to form nanoparticle drug delivery systems (nanoDDSs) with an average size of 142 nm in diameter when combined with an excess amount of deprotonated doxorubicin in an aqueous phase. A loading content of doxorubicin is as high as 23.8% in the nanoDDS. In an *in vitro* cytotoxicity experiment, the novel nanoDDS has similar cytotoxicity as free DOX against HepG2 cells. In contrast, for the incubation with HeLa cells of the novel nanoDDS, there was no significant cytotoxicity change. In a human tumor xenograft nude mouse model, the novel nanoDDS generated higher therapeutic effect than non-targeted doxorubicin nanoparticles or free doxorubicin. Together, these results suggest that novel nanoDDS, which has improved transfection efficiency and hepatocyte specificity, may be useful for tumor therapy.

1. Introduction

Nanoparticle carriers for drug delivery system (DDS) have attracted interest for their advantages, such as better bioavailability for poorly soluble drugs, a wide potential application spectrum (oral, dermal, and intravenous), and protection of sensitive drug molecules from the environment (water and light) [1,2]. Nanoparticle carriers provide a better penetration of the particles inside the body as their size allows delivery via intravenous injection or other routes. The nanoscale size of these drug delivery systems also minimizes the irritant reactions at the injection site [1,3,4].

In recent years, there has been considerable interest in developing biodegradable nanoparticles as effective drug delivery systems [4–8]. For the application of polysaccharides for drug carriers, issues of safety, toxicity and availability are greatly simplified. Nanoparticle DDS, which are made of bioadhesive polysaccharide amphiphiles, are highly stable in aqueous environment and prolongs the residence time so that they are able to increase the absorbance of loaded drugs. Xyloglucan, obtained from tamarind seed, is composed of β-(1,4)-glucan backbone chain that partially substituted by α-(1,6)-linked xylose units. Some of the xylose residues are β-D-galactosylated at O-2. Xyloglucan functions as a drug carrier because it is a water-soluble, biodegradable, and non-antigenetic natural polysaccharide. The hydroxyls of xyloglucan can be replaced by modifiable carboxylic derivatives for the grafting of drug derivatives. It can also be covalently attached to both the parent drug and the targeting unit [9–11].

However, ideal drug delivery system is able to target and to control the drug release. Targeting will ensure high efficiency of the drug and reduce the side effects, especially when dealing with drugs that are peripheral toxicity. The peripheral toxicity limits the clinical use of the drugs such as doxorubicin (DOX), which shows a valuable broad spectrum of antitumor activity. The reduction or prevention of side effects can also be achieved by controlled release. Nanoparticle carriers gain access to the tumor interstitium through the leaky tumor vasculature. The primary mechanism for the carrier method relies on diffusion, rather than convection. To further improve the delivery of the therapeutic payload, the nanoparticle carriers may be engineered to contain tumor-targeting ligands that guide the delivery of these vectors to tumor, endothelial, and potentially stromal cells. Receptor-mediated targeting is a promising approach to selective drug delivery. Tumor-targeting nanoparticle carriers assist tumor cells in the uptake of the drug by receptor-mediated endocytosis or photolysis. One wonderful example exploits the mechanisms of sugar recognition that specific cell types possess. Targeting to liver parenchymal cells is

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feasible due to the abundance of the asialoglycoprotein receptor (ASGP-R) which is specific for hepatocytes. Naturally, ASGP-R recognizes and internalizes glycoproteins bearing terminal galactose or N-acetylglucosamine residues via clathrin-coated pits. Delivery of drugs using substances bound to appropriate glycoproteins, lactose or galactose in a specific manner would provide significant therapeutic benefits in hepatic disease [12].

In the present study, the novel nanoDDS self-assemble from free DOX combined with xyloglucan grafted with DOX and galactosamine. The cytotoxicity effect of the DDS against HepG2 and HeLa human tumor cells and the therapeutic effect on HepG2 tumor cells implanted in mice were also investigated.

2. Materials and methods

2.1. Materials

Xyloglucan was prepared from tamarind seed polysaccharide. Xyloglucan was purified by precipitation from its aqueous solution into 2-propanol and dried with acetone in air prior to enzymatic hydrolysis. Purified xyloglucan (1 g) was dissolved in 100 ml of 20 mM sodium acetate buffer (pH 4.0) and incubated at 37 °C with 1 mL β-glucanase from *Trichoderma viride* (Ultraflo L, Novozymes A/S. Co. Ltd.). GPC (Prominence GPC, Shimadzu Co., Japan) was employed to follow the digestion progress. The reaction mixture was heated at 95 °C for 20 min to inactivate the enzyme and centrifuged at 8000 rpm for 15 min to remove insoluble materials. The supernatant was lyophilized. The molecular weight of xyloglucan was about 10^6 by the measurement of GPC.

Doxorubicin hydrochloride (DOX·HCl) was from Zhejiang Hisun Pharmaceutical, China. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from the Sigma–Aldrich Co., USA. All other chemicals were analytical grade unless otherwise stated. Organic solvents were purified and dried through common standard methods.

2.2. Synthesis of the carboxypentyl xyloglucan

Xyloglucan (1 g) was dissolved in 4 M NaOH solution (10 ml) and 6-bromohexanoic acid (2.8 g) was added. The reaction was kept at about 80 °C for 3 h with gentle stirring. The spacer-introduced polysaccharide, carboxypentyl xyloglucan, was dialyzed against water and freeze-dried. The white product was identified using FTIR (PE Spectrometer, KBr plate).

2.3. Conjugation of DOX and galactose to xyloglucan

For the conjugation of DOX and galactosamine to xyloglucan, carboxypentyl xyloglucan (1 g), doxorubicin (110 mg), and galactosamine (50 mg) were mixed and stirred in 200 ml of purified water, and the pH of tie mixture was adjusted to 6 using HCl (1%, w/w). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (0.8 g) was then added and adjusted to the pH of 8 with HCl. The solution was under reaction by stirring for 2–3 days at room temperature. After filtration with 0.45 μm filter membrane, the solutions were directly put into dialysis membrane (MWCO 12,000–14,000 Da) and dialyzed against excess deionized water for 4 days at room temperature. Final products of the xyloglucan conjugates were obtained by freeze-drying. The content of DOX in conjugates was determined using the absorbance at 254 nm by UV–visible spectroscopy [13]. A calibration curve was made by detecting different concentrations of DOX solution at 254 nm. The absorption of conjugate was measured at 254 nm and then the DOX content of conjugate was found by comparison with the calibration curve of DOX.

2.4. Preparation and characterization of nanoDDS

To fabricate self-organized nanoparticles, the sample (100 mg) was dissolved in 10 mL DMSO and dialyzed (MWCO 8000 Da) against excess deionized water at 4 °C for 3–4 days exchanging the water at 8 h intervals [7,13,14]. After dialysates were collected and filtrated with 0.45 μm membrane, the dialysates were freeze-dried for 3 days to obtain the nanoparticles, which could be redispersed in PBS to form the nanoparticles solution without precipitate.

To load DOX into nanoparticles, 15 mg DOX was dissolved in anhydrous 10 mL of DMSO containing triethylamine (molar ratio of triethylamine to doxorubicin = 2:1). After adding conjugate (50 mg) into the solution, the mixture was stirred overnight at dark cold room. The solution was dialyzed (MWCO 8000 Da) against excess deionized water at 4 °C for 3–4 days exchanging the water at 8 h intervals. Nanoparticles spontaneously formed by directly dispersing the organic phase into the aqueous phase. Unencapsulated doxorubicin and triethylamine were removed by extensive dialysis against deionized water. The novel nanoDDS was filtrated with 0.45 μm membrane, centrifuged, separated and lyophilized for 3 days.

Nanoparticle size and its distribution were measured by dynamic light scattering (DLS; Zetasizer 3000, Malvern Instruments LTD, UK). Transmission electron microscopy (H-600, Hitachi Ltd., Japan) was also conducted at the accelerating voltage 200 keV in order to observe the nanoparticles.

2.5. In vitro cytotoxicity assay against cancer cells

The cytotoxicity of the samples was measured against human liver cancer cell line (HepG2) and cervical cancer cell line (HeLa) with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenytetrazolium) assay [13]. Fifty microliters of the cell suspension of 1.85×10^5 cells/mL were seeded into wells of a 24-well plate. Cell viability was determined after the treated cells were incubated for 72 h. In brief, 10 μl MTT was added to the wells, the cells were cultured for 3 h at 37 °C in an incubator with an atmosphere of 5% CO_2. Then 100 μL of 10% SDS was added to the wells and the cells were cultured overnight. The formation of formazan was measured at 570 nm using microplate spectrophotometer. The inhibitory rate was calculated as follows:

$$\text{Inhibitory rate}\,(\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

2.6. In vivo cytotoxicity of nanoDDS against HepG2 cells in mice

Specific pathogen-free grade male BALB/c/nu naked mice (Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 4 weeks old, 20–30 g) were inoculated subcutaneously with human HepG2 cells (1×10^7 cells/animal). Following the implantation, tumors were allowed to grow until 3 weeks until well established. Xyloglucan–DOX nanoparticles, novel nanoDDS, or free doxorubicin (equivalent dose of doxorubicin = 5 mg/kg) suspended in PBS were injected to tail veins of animals once a week for 3 weeks. The mice were killed by vertebral dislocation after therapy for 4 weeks. A major axis and a minor axis of tumors were measured using a caliper. Tumor volume was then determined. All animals were accommodated in a pathogen-free laboratory environment throughout the experiments.

2.7. Statistical analysis

Data was expressed as means ± standard deviations of multi replicated determinations, and followed by the Student's *t*-test. Differences were considered to be statistically significant if $P < 0.05$.

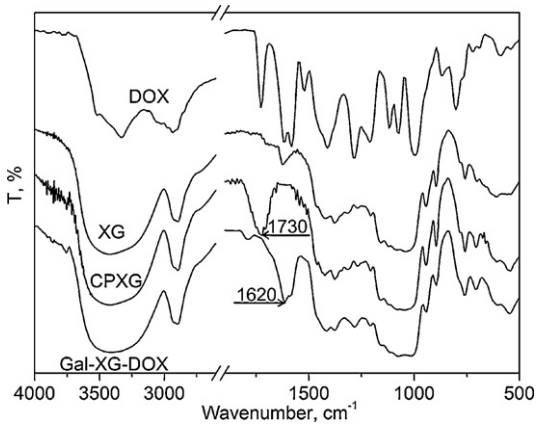

Fig. 1. FTIR spectra of DOX, xyloglucan (XG), carboxy carboxypentyl xyloglucan (CPXG) and galactosed xyloglucan–DOX (Gal-XG-DOX) conjugate.

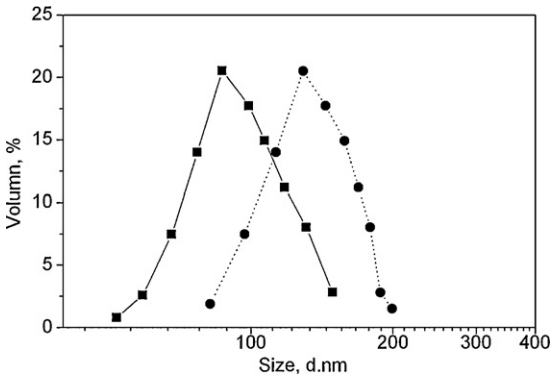

Fig. 2. Dynamic laser scattering (DLS) results of the samples (■) galactosed xyloglucan–DOX nanoparticles and (●) novel nanoDDS.

3. Results and discussion

3.1. Preparation of the galactosed xyloglucan–DOX conjugate

Xyloglucan, which is a water-soluble, biodegradable, and non-antigenetic natural polysaccharide, has many essential properties to be a polymeric carrier in drug delivery system (DDS). However, the hydroxyls of polysaccharide cannot connect with drug directly, which is required to replace with modifiable carboxylic derivatives before being coupled with the drug. In this study, the activation of xyloglucan was conducted by mixing polysaccharide with 6-bromohexanoic acid in alkali condition to produce the corresponding carboxypentyl moiety. The product was identified in Fig. 1. The signal about 1730 cm^{-1} was attributed to the stretching of C=O and is wide and strong in the FTIR spectral of carboxypentyl xyloglucan. The anthracycline antibiotic, doxorubicin (DOX), is one of the most powerful antitumor drugs in the field of cancer chemotherapy, and presently used for the clinical treatment of a broad range of human malignancies, such as leukemia and cancer of the liver, ovary, and breast. The amino and carbonyl groups of DOX were suitable for grafting the drug to carriers by amide bond and hydrazone bond, respectively. The attachment of DOX and galactosamine to the carboxypentyl xyloglucan was accomplished using EDC as the coupling reagent under aqueous conditions. The peak about 1620 cm^{-1} might be perhaps due to the attraction of the electrons by the conjugated DOX.

The content of DOX, determined by detection and calculation via UV spectra, was about 5% (wt). In this study, we found that the DOX content in the conjugate rose with the increase in the molar ratio of DOX/xyloglucan. Hydrophilicity of polysaccharide was adjusted by the control of the content of DOX or carboxypentyl. The final DOX content in the conjugate was about 5% (wt) in the present study, because the conjugates with the high DOX content have negative influence on the stability and the size of nanoparticles.

3.2. Self-organization and characterization of nanoDDS

Self-organized nanoparticles of modified polysaccharide amphiphiles have been intensively investigated in the biomedical and pharmaceutical fields due to their potential based on biocompatibility and abundance [4,13,15,16]. It is well known that nanoparticles consist of hydrophobic core and hydrophilic shell to make zero Gibb's energy and are able to enclose a hydrophobic drug in core and to protect interactions with biological environment. The DOX and carboxypentyl have hydrophobic characteristics, and hydrophobic groups were also introduced to a hydrophilic xyloglucan molecule. As the conjugates have amphiphilic structure elements within the molecules, these conjugates could possibly form self-organized nanoparticles. The self-organized nanoparticles were characterized by the DLS technique. Xyloglucan, used as the starting material, had a mean diameter of 16.5 nm. The result indicated that the polysaccharide without any chemical modification does not form self-aggregates. The mean particle diameters of the galactosed xyloglucan–DOX conjugates self-aggregated nanoparticles in the range from 50 to 150 nm (Fig. 2), which were apparently greater than that of xyloglucan.

The galactosed xyloglucan–DOX conjugate with high doxorubicin content was precipitated in aqueous media due to excessive hydrophobicity. For increasing the DOX content in the formulations, free doxorubicin was physically loaded into the hydrophobic core of galactosed xyloglucan–DOX nanoparticles. Doxorubicin was successively entrapped physically into the nanoDDS. The amount of entrapped doxorubicin reached 23.8% and was more than four times the amount of chemically conjugated doxorubicin. After entrapping, the size of the nanoparticles increased to the

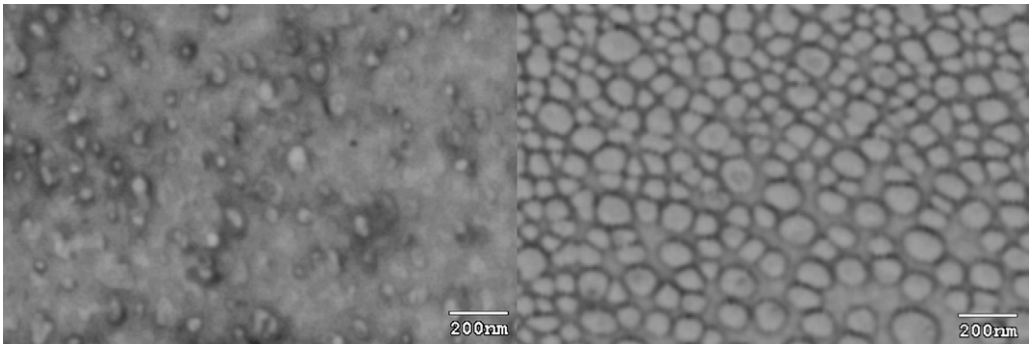

Fig. 3. TEM micrograph of the nanoDDS (Left, galactosed xyloglucan–DOX nanoparticles. Right, novel nanoDDS).

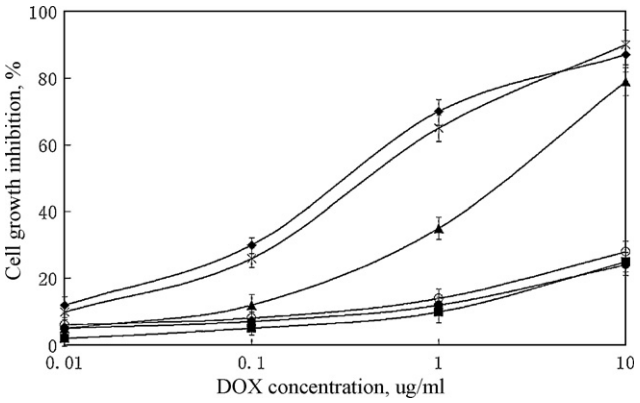

Fig. 4. Antitumor activity *in vitro* of novel nanoDDS (×), DOX (♦), xyloglucan–DOX nanoparticles (●), galactosed xyloglucan–DOX nanoparticles(▲) against HepG2 cell and galactosed xyloglucan–DOX nanoparticles against HeLa cell(■) and nanoDDS loaded DOX against HeLa cell(○).

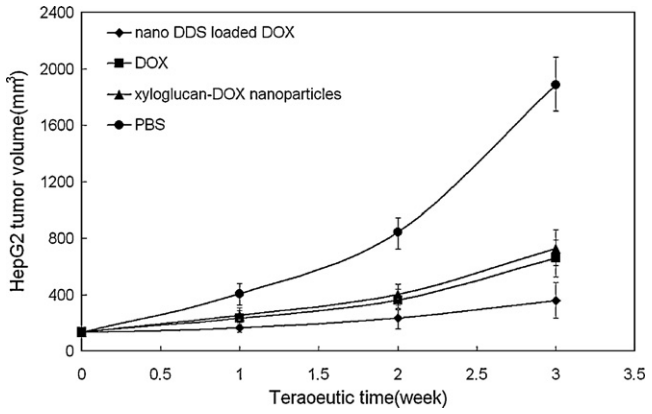

Fig. 5. Antitumor activity *in vivo* of novel nanoDDS (♦), DOX(■) and xyloglucan–DOX nanoparticles (▲) against HepG2 cell.

range from 80 to 200 nm and the mean diameter of nanoparticles is about 142 nm in aqueous media (Fig. 2). This showed that free doxorubicin was inserted into galactosed xyloglucan–DOX nanoparticles. This physical entrapment efficiency was dependent on the hydrophilic–hydrophobic balance and core structure of the galactosed xyloglucan–DOX nanoparticles.

The TEM images of nanoparticles were also observed in Fig. 3, which is not well consistent with the DLS results. The differences in size between the dried and swollen nanoparticles resulted from the huge hydrodynamic volume of the nanoparticles.

NanoDDS can be used for cancer therapy without any targeting moieties. In this case, the therapeutic effect can be achieved by a specific pathophysiology of cancer, named as enhanced permeability and retention (EPR) effect. It has been reported that solid tumors show hypervascular permeability and impaired lymphatic drainage [8]. As a result, the nanoparticles, which the size is less than 200 nm, could significantly accumulate in tumor by "filtration" mechanism.

3.3. *In vitro cytotoxicity of the nanoDDS against tumor cells*

The cytotoxicity effect of these nanoDDS was determined by MTT assay against HepG2 and HeLa cells. HepG2 and HeLa cells were cultured for 72 h under exposure to the samples. The results for MTT assay are shown in Fig. 4. The novel nanoDDS and free DOX show the highest growth-inhibitory effect against HepG2 cells among the samples. Galactosed xyloglucan–DOX nanoparticles shows less bioactivity for HepG2 cell than free DOX, but the nanoparticles and nanoDDS show no significant growth inhibition effect towards HeLa cell. Compare with two targeting nanoDDS, xyloglucan–DOX conjugate nanoparticles shows low cytotoxicity against HepG2 cell in experiment concentration.

The results on MTT assay was decide by the structure of the samples. Free DOX shows good growth-inhibitory effect against tumor cells, because DOX can be readily transported into cells by the mechanism of passive diffusion without driven energy. The asialoglycoprotein receptors (ASGP-Rs) are present at a high density only on hepatocytes, and are retained on several human hepatoma cell lines. There are about 500,000 receptors per cell. ASGP-R internalizes the glycoprotein by receptor-mediated endocytosis (RME), by which cells bind macromolecules through receptor recognition [17,18]. Thus, the galactosamine residues in DDS are usually utilized as the targeting moiety, based on the ability of acutely targeting at the surface of the hepatoma cell. In this study, the higher cytotoxicity effect of targeting nanoDDS than xyloglucan–DOX nanoparticles are due to their faster rate of cellular uptake. Compare with galactosed xyloglucan–DOX nanoparticles, novel nanoDDS released rapidly numerous DOX in cell. There is no ASGP-R at the surface of HeLa cells, which are a carcinoma cell line of the cervix. Hence, two targeting nanoDDS have similar cytotoxicity effects without a significant effect of HeLa cell killing.

3.4. *In vivo antitumor activity of nanoDDS*

The *in vivo* cytotoxicity effect of free DOX, xyloglucan–DOX nanoparticles, and novel nanoDDS was examined in order to compare their ability to suppress the growth of tumor cells in Balb-C nude mice. The intravenous route through the tail vein was selected for the injection of free DOX or nanoDDS into tumor-bearing mice. Results are summarized in Fig. 5. By comparison of the volume of the tumor in control and drug-treated group, tumor volume for mice treated with novel nanoDDS was about 45% less than that of those treated with free DOX. These results demonstrate that novel nanoDDS have a superior antitumor activity to free DOX and xyloglucan–DOX nanoparticles in terms of tumor inhibition, while xyloglucan–DOX nanoparticles showed fair more *in vivo* antitumor activity than that of free doxorubicin. The present dosage cannot completely inhibit the growth of the tumors by any of the conjugates in the experimental period. This is possibly due to an insufficient drug dosage.

The novel nanoDDS loaded DOX increased *in vivo* anti-cancer effects of DOX with a statistical significance ($p < 0.05$). The targeting group improved antitumor effect of the nanoDDS: passive targeting and active targeting. The passive targeting allowed nanoparticles into the tumor tissue, while the active targeting permitted them to be readily taken up by hepatocyte tumor cells at the site. Thus the combined passive and active targeting effects were likely to act synergistically, and they were mainly responsible for the observed delayed tumor volume growth. Since DOX molecules were slowly released out in an aqueous phase from nanoDDS, a small amount of DOX could be continuously released out in the tumor tissue. This sustained release DOX at the target site would take an additional advantage in decreasing the tumor volume. The present study suggests that galactosed xyloglucan–DOX conjugate could serve as a novel amphiphilic polysaccharide derivative to stabilize unprotonated DOX aggregates in a nanoparticle and it is suitable for targeting to the tumor tissue, enhancing cellular uptake, and achieving sustained release within cells or targeting tissue. Further studies are underway to optimize the conjugate content and the drug dosage, so that the antitumor function of the loaded drug can be exerted maximally.

4. Conclusion

The objectives of the current study were to develop a simple fabrication method of self-organized nanoparticles holding both anti-cancer drug and targeting moiety. Modification successfully produced polysaccharide amphiphile and it spontaneously formed self-organized nanoparticles in dialysis. The novel nanoDDS self-aggregated from the conjugates combined with an excess amount of deprotonated DOX in an aqueous phase, in which the content of doxorubicin reached 23.8%. The competition studies supported that the novel nanoDDS showed targeting and effective in HepG2. In summary, it is reasonable to propose that the novel nanoDDS can be used for active targeting chemotherapy.

References

[1] C. Pinto Reis, R.J. Neufeld, J. Antonio, F. Veiga, Nanomed. Nanotechnol. Biol. Med. 2 (2006) 8–21.
[2] K. Na, T. Bum Lee, K.H. Park, E.K. Shin, Y.B. Lee, H.K. Choi, Eur. J. Pharm. Sci. 18 (2003) 165–173.
[3] M. Sletmoen, G. Maurstad, B.T. Stokke, Food Hydrocolloid 22 (2008) 2–11.
[4] M.J. Vicent, R. Duncan, Trends Biotechnol. 24 (2006) 39–47.
[5] A. Dufresne, Can. J. Chem. 86 (2008) 484–494.
[6] G.F. Payne, S.R. Raghavan, Soft Matter 3 (2007) 521–527.
[7] F.L. Mi, Y.Y. Wu, Y.L. Chiu, M.C. Chen, H.W. Sung, S.H. Yu, S.S. Shyu, M.F. Huang, Biomacromolecules 8 (2007) 892–898.
[8] I. Bertholon, H. Hommel, D. Labarre, C. Vauthier, Langmuir 22 (2006) 5485–5490.
[9] T. Coviello, P. Matricardi, F. Alhaique, Expert Opin. Drug Deliv. 3 (2006) 395–404.
[10] S. Burgalassi, P. Chetoni, L. Panichi, E. Boldrini, M.F. Saettone, J. Ocul. Pharmacol. Ther. 16 (2000) 497–509.
[11] F. Suisha, N. Kawasaki, S. Miyazaki, M. Shirakawa, K. Yamatoya, M. Sasaki, D. Attwood, Int. J. Pharm. 172 (1998) 27–32.
[12] K. Kunath, A. von Harpe, D. Fischer, T. Kissel, J. Control. Release 88 (2003) 159–172.
[13] K.A. Janes, M.P. Fresneau, A. Marazuela, A. Fabra, M.J. Alonso, J. Control. Release 73 (2001) 255–267.
[14] C. Lemarchand, R. Gref, P. Couvreur, Eur. J. Pharm. Biopharm. 58 (2004) 327–341.
[15] A.W. Pan, B.B. Wu, J.M. Wu, Chin. Chem. Lett. 20 (2009) 79–83.
[16] S. Kim, K.M. Park, J.Y. Ko, I.C. Kwon, H.G. Cho, D. Kang, I.T. Yu, K. Kim, K. Na, Colloid Surf. B 63 (2008) 55–63.
[17] L. Dong, S.Y. Gao, H.J. Diao, J.N. Chen, J.F. Zhang, J. Biomed. Mater. Res. Part A 84A (2008) 777–784.
[18] A. Murao, M. Nishikawa, C. Managit, J. Wong, S. Kawakami, F. Yamashita, M. Hashida, Pharm. Res. 19 (2002) 1808–1814.