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Chitosan-functionalized poly(lactide-co-glycolide) nanoparticles: ultimate breaking through the brain's tight security gateway

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Despite the development of several progressive treatments, many effective anticancer drugs have failed in clinical examinations due to the inability to penetrate across the blood-brain barrier. For this reason, the local and controlled delivery of anticancer drugs using biodegradable polymeric nanoparticles is attracting increasing attention as a viable therapy for treating brain tumors. In the present study, poly(lactide-co-glycolide) (PLGA) nanoparticles
Q4 **(NPs) with high emulsifying effects were prepared in conjunction with vitamin E D-α-tocopheryl polyethylene glycol 1000 succinate (TGPS) and chitosan, which imparted the desired surface morphology and particle size. The particle sizes, the surface morphology and the phase composition were correlated with the loaded amount of chitosan and operating pH. The laboratory cytotoxicity of the particles was investigated using the PC12 cell line. An increase in the chitosan content decreased the particle sizes of the NPs in a semilinear pattern and increased the potential therapeutic effects as indicated by high cell viability. These chitosan surface-modified PLGA NPs could be used for local drug delivery, thereby greatly expanding the spectrum of drugs available for the treatment of brain tumors.**

1. Introduction

Drug therapy represents an important means of combating a wide variety of neurological disorders, ranging from brain tumors to Parkinson's disease. The administration of therapeutic drugs to the central nervous system is complicated due to the presence of the blood-brain barrier. The same mechanisms that protect the brain from foreign substances unfortunately also restrict the entry of many potentially therapeutic agents.[1] However, the introduction of drug delivery carriers that are capable of crossing the blood-brain barrier now provides the possibility of achieving high concentrations of therapeutic drugs within brain tissues[2,3] while simultaneously reducing systemic toxicities commonly encountered with intravenous administration.[4] A number of drug delivery systems, including liposomes, emulsions, micelles and polymeric micro/nanoparticles, have shown potential for controlling the rate and the duration of drug delivery and/or targeting the drug to specific cells or tissues.[5,6]

Particularly, the development of targeted drug delivery systems for brain cancer therapy has become an important area of research. According to the World Health Organization (WHO), cancer is a leading cause of death worldwide. From a total of 58 million deaths in 2005, cancer accounts for 7·6 million (or 13%) of all deaths.[7] The WHO report also reveals that cancer has emerged as a major public health problem in developing countries, matching its effect in industrialized nations.[8] The principal modes of cancer management are surgery, radiotherapy and chemotherapy.[7] Chemotherapy, the use of cytotoxic drugs to kill cancerous cells, remains the most common approach for cancer treatment. Generally, cytotoxic drugs are highly toxic but

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poorly specific; that is, they do not differentiate between normal and cancer cells.[7] A further complication is the occurrence of multiple drug resistance, which seriously reduces the efficacy of many chemotherapeutic agents for cancer. P-glycoprotein, an efflux pump overexpressed on the cell surface, plays an important role in drug resistance. Several surfactants, such as vitamin E D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS), can inhibit P-glycoprotein,[9] making these attractive compounds for incorporation into anticancer therapies.

Anticancer drug carriers have become the subject of intensive research in the past decades and now represent an important area in cancer nanotechnology. These carriers are capable of accumulating inside tumors after systemic administration, with a biodistribution largely determined by their intrinsic physical and biochemical properties. These same properties also affect their mode of action and their interactions with both the drugs and the tumors.[10] Biodegradable polymeric carriers can provide a way to sustain, control and target the delivery of formulated drugs to improve their therapeutic effects and reduce their side effects.[10,11] Among these, polymeric nanoparticles (NPs) are promising candidates since, due to their small size and chemical nature, they are capable of (*a*) opening the tight junctions of the blood-brain barrier, (*b*) effectively disguising the membrane barrier limiting the characterizations of the drug molecule, (*c*) prolonging the drug release and (*d*) protecting against enzymatic degradation.[11,12] In fact, the brain distribution of many inherently impermeable drugs has been improved following the incorporation into polymer-based NPs.[13]

Poly(D,L-lactide-co-glycolide) (PLGA) is one of the most widely used polymers for the production of functional NPs. These NPs are designed to sustain,[14] target[15] and localize[16] the delivery of various agents, including anticancer drugs,[17] plasmid DNA,[18] proteins and peptides[19] and low-molecular weight compounds.[20] PLGA NPs have shown great efficiency as drug delivery vehicles due to their ability to increase the amounts of drugs crossing various biological barriers, including the blood-brain barrier,[21] as well as gastrointestinal mucosa,[22] nasal mucosa[23] and ocular tissue.[24]

The main drawback of PLGA NPs is their non-specific interaction with cells and proteins, which leads to drug accumulation in non-target tissues. They also lack suitable functional groups that would allow efficient covalent conjugation with bioactive ligands. Therefore, physical and chemical surface modifications of PLGA NPs have been attempted to overcome these limitations.[25] However, PLGA-based NPs are usually produced by a solvent evaporation/extraction technique, and a number of properties of the NPs can be altered through the manipulation of the processing parameters. One of these parameters is the use of emulsifiers as surfactant stabilizers.[6]

The cationic surface modification of PLGA NPs can overcome many of the existing drawbacks, and several polycations have been tested for this purpose, including polyethyleneimine,[26] cetyltrimethylammonium bromide,[27] poly(2-dimethylamino) ethyl methacrylate,[28] didodecyldimethylammonium bromide[29] and chitosan.[30]

Chitosan, a naturally occurring linear polysaccharide, is viewed as a good surface-modifying candidate by virtue of its biodegradability, biocompatibility, mucoadhesiveness and permeability-enhancing properties.[31] The chemical structure of chitosan is illustrated in Figure 1.

A recent comparative study confirmed that chitosan-coated NPs were well suited for transfection.[24] In addition, many studies have reported a relatively high positive charge for chitosan and have demonstrated in the laboratory cytotoxic effects against various human tumor cell lines.[32] These studies have revealed many advantages for chitosan-modified NPs, such as their bioadhesiveness and prolonged drug release properties when compared to unmodified NPs.[32] Low- and medium-molecular weight chitosans (1–300 kDa) also show biological activities, such as antiinflammatory, antitumor, antifungal and antimicrobial effects.[33–35]

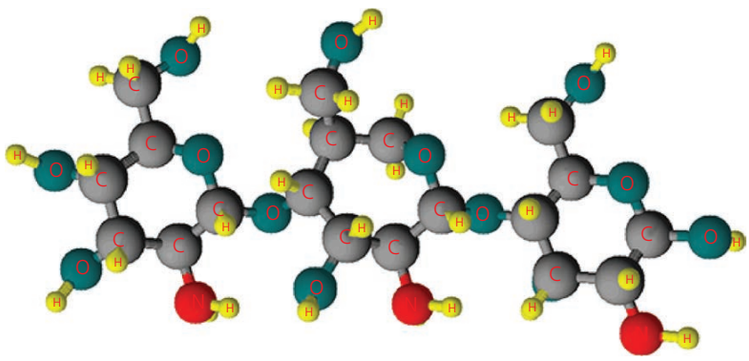

Figure 1. Three-dimensional model of the typical chemical structure of chitosan molecules

Chitosan has a positive charge in acidic solutions due to the presence of protonated amino groups along its backbone.[36] The cell surfaces, by contrast, usually carry a net negative charge due to the translocation of negatively charged constituents of the inner layer of the cell membrane (e.g. phosphatidylserine, anionic phospholipids, glycoproteins and proteoglycans) to the cell surfaces; this is especially amplified in cancer cells.[37,38] Thus, chitosan-coated NPs can be preferentially targeted to the surfaces of cancer cells. The chitosan coating on the surface of NPs can also significantly reduce the initial burst of drug release from the NPs, without affecting other parts of the release profile.[39]

In the present work, the authors studied the potential of the surface modification of PLGA NPs with chitosan molecules. The predicted advantages of modifying PLGA particle surfaces with the mucoadhesive chitosan polymer were (*a*) decreased burst effect for the encapsulated drugs, (*b*) increased stability of macromolecules such as proteins, (*c*) reversal of the zeta potential, which would promote cell adhesion and retention of the delivery system at the target site and (*d*) conjugation of targeting ligands to the free amine groups on chitosan.[40]

The purpose of the present study was to investigate the effect of PLGA surface modification with chitosan on the physicochemical properties of PLGA NPs, including particle size, morphology and especially their molecular interactions and cell responses.

2. Materials and method

2.1 Preparation of chitosan surface-modified PLGA NPs

The NPs were prepared by dissolving 50 mg of PLGA (50:50, Resomer-RG 503H; Boehringer Ingelheim) in 1 ml of dichloromethane (DCM). Different percentages of chitosan (medium molecular weight of 190 000–310 000, Orbital Pharma Co. (Hebei, China)) were separately dissolved in 1 ml acetic acid (98%, Merck), followed by the addition of 20 ml distilled water, in 2 ml aliquots, with stirring. The solutions were then poured into 30 ml aqueous solutions of 0·12% TPGS (Sigma-Aldrich). The NPs were prepared by a one-step oil-in-water emulsion under moderate magnetic stirring to evaporate the DCM. The particles prepared by this method were not washed since the components of the NPs were all helpful for neural applications. The final product was obtained after freezing for 2 d and then freeze-drying for 48 h.

2.2 Characterization

2.2.1 Particle size and morphology

The particle size and the morphology of the synthesized NPs were evaluated using a scanning electron microscope (SEM; Philips XL30) operating at the acceleration voltage of 15 kV. The samples were coated with a thin layer of gold (Au) by sputtering (Emitech K450X, England) before observation.

A better observation at higher magnifications was obtained using a transmission electron microscope (TEM). Before the observation, the NPs were dispersed in ethanol using an ultrasonic apparatus (Sonicator 3000, Misonix) for 45 min. The drops of ethanol containing the nanoparticles were then poured onto the surface of a carbon-coated copper mesh disk. The NPs were observed with an electron microscope (Zeiss (Germany), EM10c, 80 kV, TEM).

2.2.2 Chemical bonding

The Fourier transform infrared spectrometer (FTIR; Bomem MB series) spectra of the samples were obtained using the reflection absorption spectroscopy technique involving the use of an all-reflective objective lens while simultaneously viewing under a ×10 eyepiece. Briefly, the samples for the FTIR analysis were prepared by grinding 90% potassium bromide (KBr) (infrared-grade) with 10% of the NPs and then pressing the mixture into a transparent tablet. A total of 20 scans were coadded for each sample at a resolution of 4 cm^{-1}, and the spectra were recorded from 400 to 4000 cm^{-1}. The spectral data were collected using the Bomem software, and the numerical values were transferred to Microsoft Excel software for graphical representation.

2.2.3 Laboratory cytotoxicity studies in the PC12 nerve cell line

The laboratory cytotoxicity of the synthesized NPs containing different amounts of chitosan was tested using the PC12 cell line. The line was kept in a continuous culture in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum and 100 U/ml streptomycin/penicillin (1%). The cells were detached with trypsin/ethylenediaminetetraacetic acid before seeding onto the samples. For seeding, the cells were trypsinized, centrifuged and resuspended in a complete culture medium. A culture volume containing 90 000 cells was seeded in each well of a 24-well plate, and 6 mg/ml of the prepared NP samples were added to each well. After 72 h, 400 µl of the medium was discarded from each well, the cells were incubated with the samples for 72 h, and then 100 µl of the MTT solution (5 mg/ml) was added into each well. After incubation for 12 min–2 h, all the media were discarded, and 600 µl of dimethyl sulfoxide was added to each well. The contents of each well were then aliquoted into a 96-well plate and shaken for 10 min. After 30 min, the optical density was read (590 nm with a reference filter of 620 nm).

2.2.4 Blood-brain barrier transport study

In the blood-brain barrier transport study step, six groups of mice received intravenous tail-vein administration of chitosan surface-modified PLGA NPs, which had been previously loaded with coumarin-6 using a procedure detailed before. Briefly, 0·1% coumarin-6 was added to the DCM solution, and the obtained nanoparticles were passed through a 1·5 × 20 cm Sepharose column to remove the unentrapped coumarin-6. After injection (injection dose of 30 µg/kg in mice), the mice were sacrificed at different time intervals, the brains were collected immediately and washed twice with normal saline solution, and the coumarin-6 concentration was measured.

2.3 Statistical analysis

All the experiments were performed in five replicates. The results were given as means ± standard error. The statistical analysis was performed by using one-way analysis of variance, and Tukey's tests were reported as significant when $P < 0{\cdot}05$. The group normalization was investigated using the Kolmogorov-Smirnov test.

3. Results and discussion

3.1 Particle size study

Generally, TPGS is a more effective and safer emulsifier than conventional emulsifiers, such as polyvinyl alcohol (PVA). The residual molecules of TPGS remaining on the prepared samples are not toxic and can even be helpful in particular applications.[5] Through a recent dedicated study, the authors have determined the optimum amount of TPGS necessary for the effective emulsification of PLGA NPs. Accordingly, this amount was used in the present study, in the formulation of the PLGA NP.[41] The particle size of the obtained NPs with this amount of TPGS ranged from 70 to 800 nm.

Chitosan was added to this formulation of chitosan as a surface modifier, and the authors examined the effects of various percentages of chitosan, as well as the operating pH of the

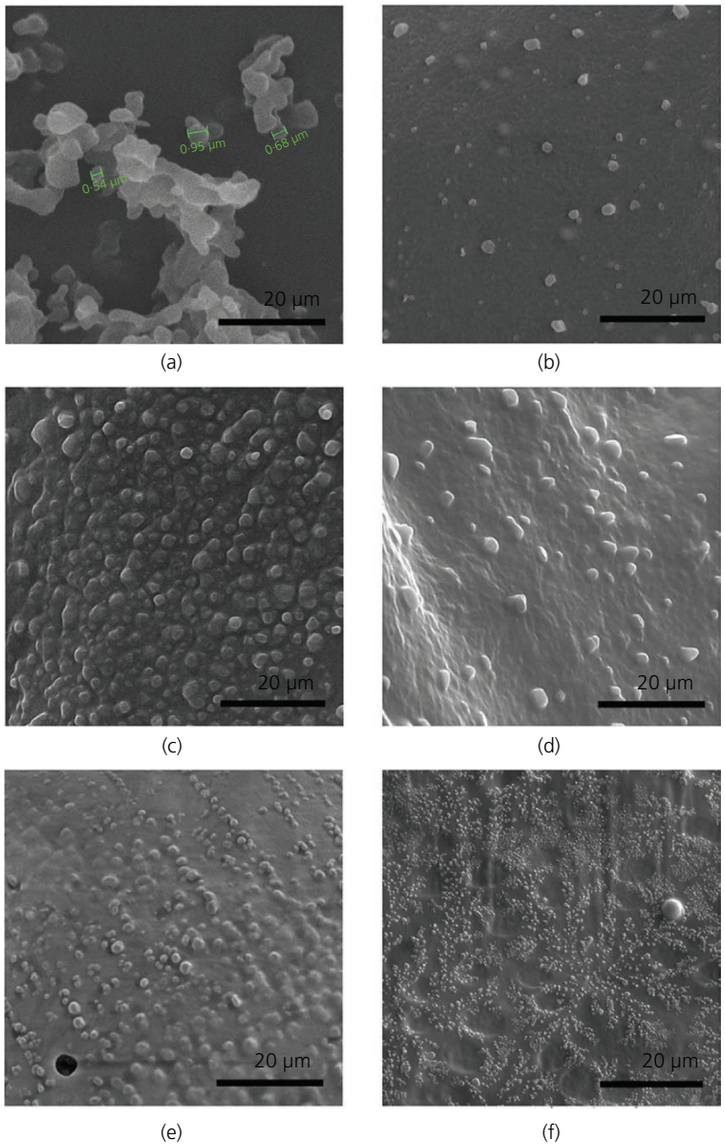

Figure 2. SEM micrographs of the chitosan surface-modified PLGA NPs containing different weight/volume percentages of chitosan: (a) 0·002, (b) 0·01, (c) 0·02, (d) 0·06, (e) 0·08 and (f) 0·1

mixture, on the surface morphology and the particle size of the PLGA NPs. The SEM micrographs of the PLGA NPs modified with different percentages of chitosan are shown in Figure 2. The PLGA particles containing small amounts of chitosan (e.g. 0·002%) had an average particle size of approximately 1 µm. The further addition of chitosan to the synthesis media induced a significant reduction in the particle size, as shown in Figure 2. A semilinear shape correlation was found between the average size of the NPs and the added amount of chitosan, as depicted in Figure 3(a).

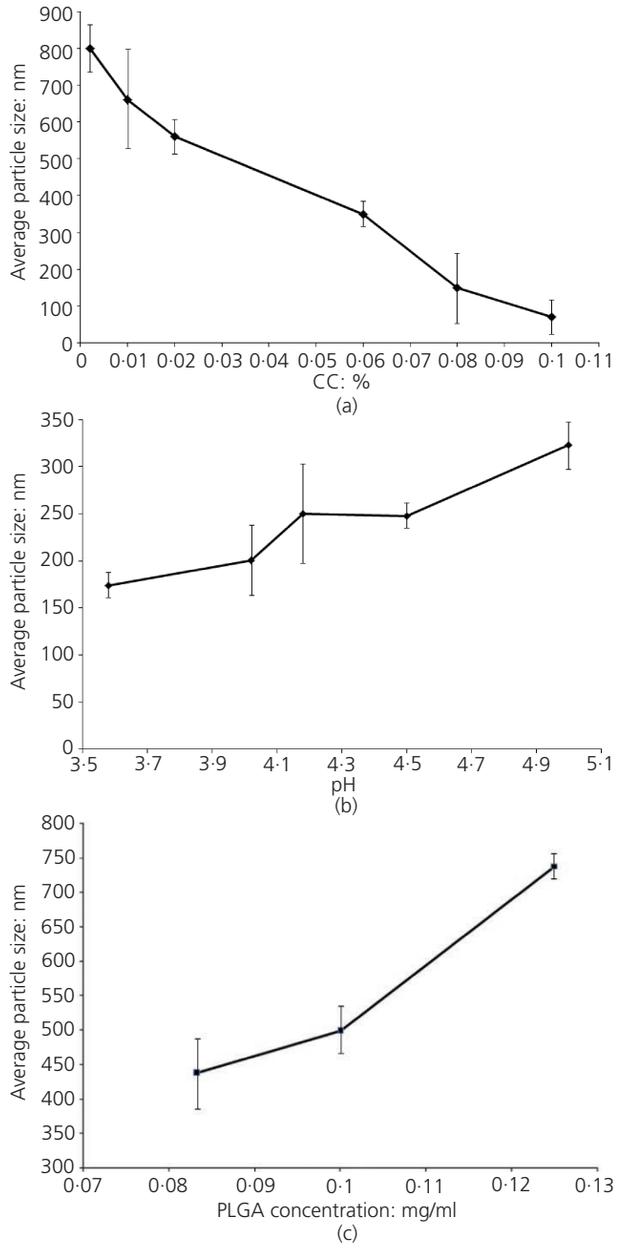

Figure 3. Effect of the polymer concentrations and different pH values on the nanoparticles size: (a) average size of modified PLGA nanoparticles versus weight/volume percentage of chitosan; (b) average size of chitosan-modified PLGA NPs at different pH values; (c) average size of nanoparticles versus PLGA concentration

According to Ravi Kumar *et al.*,[30] the electrostatic interaction between the negatively charged drugs and the $–NH_2$ groups of chitosan on the surface could be very useful for drug encapsulation, as these groups offer a higher efficiency of drug delivery. The pH is well known to influence the protonation state of the $–NH_2$ groups; therefore, the effect of the operating pH on the particle size was also studied (see Figure 3(b)) by adjusting the pH of the chitosan solution in the range of pH 3·5–5 before the preparation of PLGA NPs. The synthesis of the chitosan-modified PLGA NPs in this range resulted in different particle sizes. The targeted pH values were achieved by the stepwise addition of 0·4% w/v sodium hydroxide (NaOH) to the synthesis solution, stirring for 3 min and then readjusting the pH. Increasing the pH above 5·5 resulted in the precipitation of large amounts of chitosan due to the coagulation of the chitosan molecules in non-acidic environments. According to Mourya *et al.*,[42] chitosan is only soluble in media below pH 5·6 as the protonated free amino groups on glucosamine facilitate the solubility of the chitosan molecules under acidic conditions.[43] An increase in the average particle size, with a semilinear correlation, was observed upon increasing the pH of the solution. This suggests that the chitosan molecules tend to coagulate at higher pH values; hence, the solution is not sufficiently homogeneous for the creation of smaller particles. In contrast, at lower pH values, the less coagulated solution could create smaller particles.

The type and the mass ratio of a surfactant are known to play an important role in the preparation of the PLGA particles.[44] Figure 3(c) clearly shows that increasing the polymer

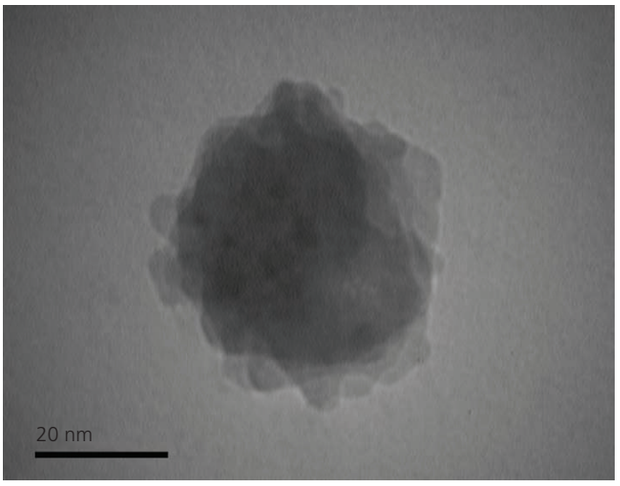

Figure 4. Structure of PLGA NPs with 0·1% (w/v) chitosan observed by TEM microscopy

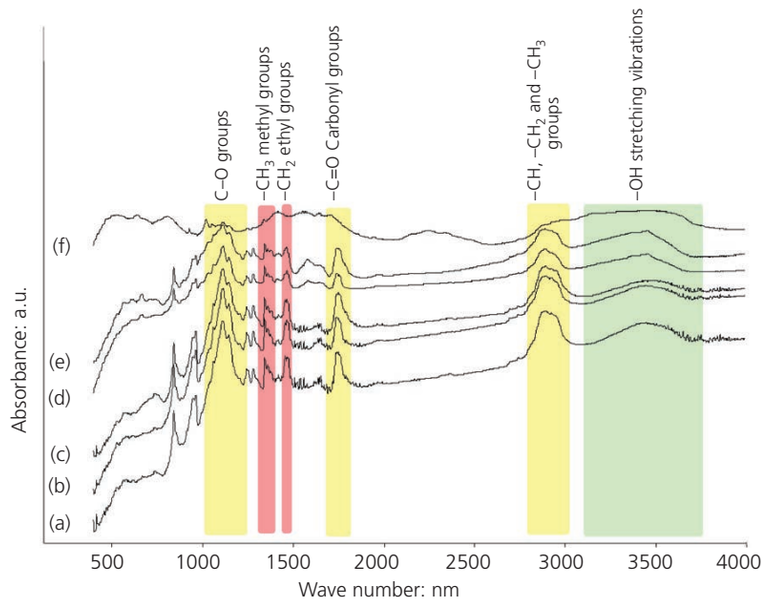

Figure 5. FTIR spectra of the chitosan surface-modified PLGA NPs containing different weight/volume percentages of chitosan: (a) 0·002, (b) 0·01, (c) 0·02, (d) 0·06, (e) 0·08 and (f) 0·1

concentration also increased the particle size, which suggested that the increased polymer concentration prevented the emulsifier from effectively acting due to the lower emulsifier –to-polymer ratio in the solution and the higher viscosity of the medium.[45]

3.2 The morphology of the synthesized particles

The micrographs in Figure 2 show particles with a semiuniform size distribution. The combined use of TPGS and chitosan for the preparation of PLGA NPs resulted in a considerable reduction in the particle size, down to 100 nm, and more importantly, it created NPs with very narrow size distribution and fewer agglomerated particles. In other words, the use of chitosan as a surface-modifying agent for the PLGA particles gave particles in the size range of 100–400 nm, confirming that an increased amount of chitosan decreased the particle size, as shown in Figure 3. These findings are in agreement with the data reported

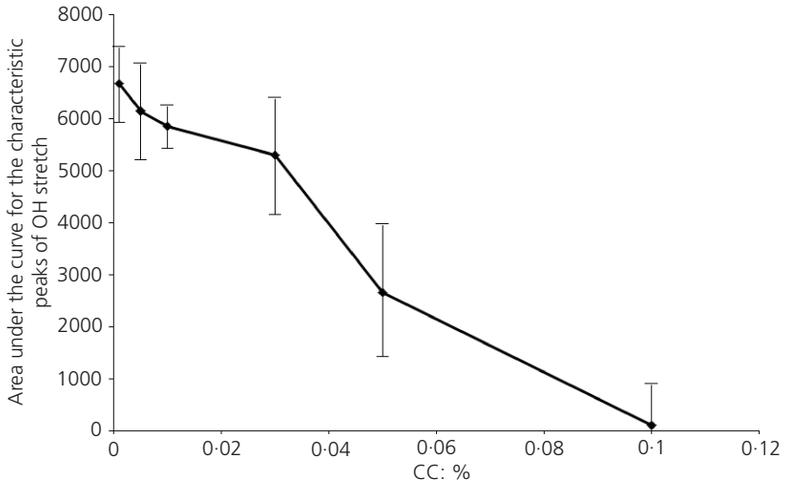

Figure 6. Variation of the area under the curve of the relative absorption intensities at 2830 and 3942 cm^{-1} as a function of the chitosan percentage in chitosan surface-modified PLGA NPs

in the literature.[46–48] The TEM observations indicated that the particles agglomerated with each other, as shown in Figure 4. These NPs stuck together to such an extent that even ultrasound treatment could not separate them; they adhered together with a powerful energy, such as physical adsorption.

One noteworthy advantage of using NPs for drug delivery to the brain precisely results from their small size. NPs penetrate into even small capillaries and are taken up within cells, allowing an efficient drug accumulation at the targeted sites in the body.[11]

3.3 Chemical bonding interactions

A better understanding of the surface chemistry of the synthesized samples was obtained using FTIR. The addition of chitosan created a clear difference in the FTIR absorbance spectra of the PLGA particles, in both the shape and the intensity of the absorption peaks. All series of the prepared particles were measured, and the obtained spectra are illustrated in Figure 5.

The samples containing different percentages of chitosan showed the characteristic absorption peaks corresponding to both PLGA and chitosan molecules. All synthesized samples showed the main peaks corresponding to the functional groups of the PLGA chemical structure, such as methine group (–CH), methylene group ($–CH_2$), methyl group ($–CH_3$) (2800–3000 cm^{-1}), carbonyl group (–C=O) (1700–1800 cm^{-1}), carboxyl group (C–O) (1000–1200 cm^{-1}), ethyl ($–CH_2$) (1400–14 700 cm^{-1}), methyl ($–CH_3$) (1200–1300 cm^{-1}) and hydroxyl group (–OH) stretching vibrations (3400–3700 cm^{-1}).[49] The notable distinction between the synthesized particles came from the absorption feature in the region of 3000–3400 cm^{-1} for chitosan modification. The diversity among various NPs could be related to this wavenumber range, although most of the absorption peaks overlapped to a large extent. All the samples exhibited typical absorption peaks of TPGS, which could be observed at 1370 cm^{-1} for methyl groups, 1250 cm^{-1} for C–O stretching of the phenol groups, and 1100 cm^{-1} for ether groups.[50] In the authors' previous publication,

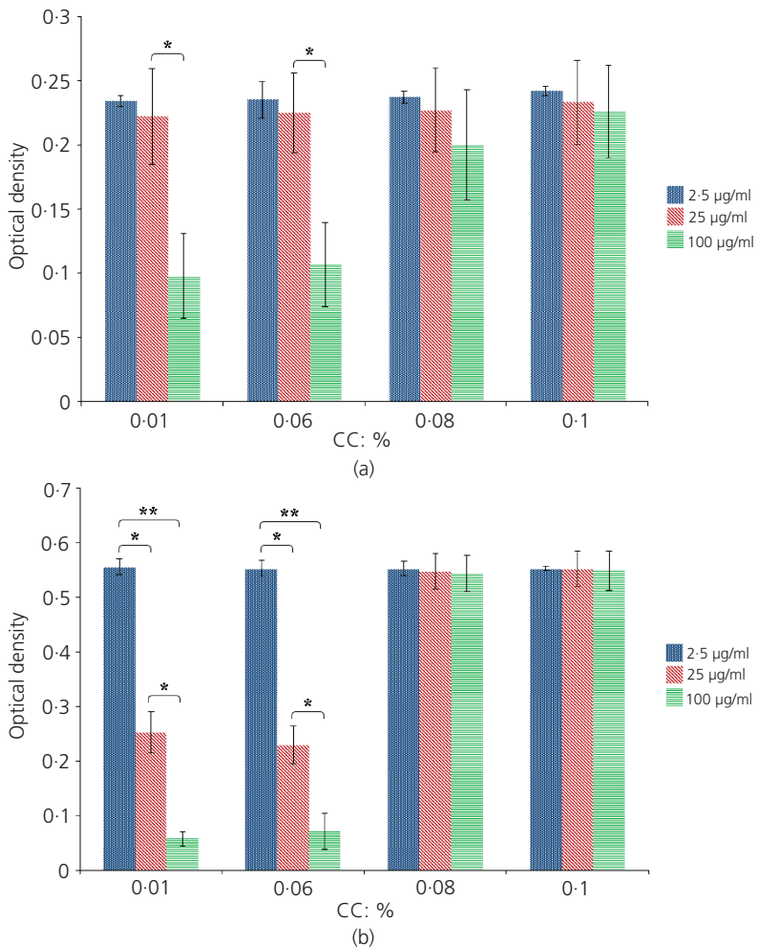

Figure 7. Effect of the chitosan-modified PLGA nanoparticles dosages and the chitosan concentration (% w/v) on the metabolic activity of PC12 cells after (a) 1 and (b) 3 d determined by the MTT assay. The nanoparticles dosages (µg/ml) are shown with different bar colors: 2·5 (blue), 25 (red) and 100 (green)

they concluded that the presence of the desired amounts of TPGS could not alter the structure of the PLGA.[41] The FTIR analysis showed the presence of the specific functional groups of both the PLGA polymer material and the chitosan molecules on the particle surfaces.[51,52]

The chitosan surface-modified samples clearly showed the major characteristic peaks around 850 and 1200 cm^{-1} related to the repeating saccharide unit of the chitosan. The sharp peaks around 1505 cm^{-1} were assigned to the methyl group symmetrical deformation mode, while the absorption peaks at 1746, 1510 and 1340 cm^{-1} were ascribed to the amide I, II and III peaks respectively. The peak at 1022 and 1218 cm^{-1} indicated the C–O stretching vibration in chitosan. The peak around 2930 cm^{-1} was attributed to the typical C–H stretch vibrations, and the broad peak around 3500 cm^{-1} was caused by amine N–H symmetrical vibration.

A more detailed study of the effect of the further addition of chitosan to the samples was conducted by calculating the area under the curve for the peaks at 2830 and 3942 cm^{-1}, as these are the characteristics of the hydroxyl group stretch and can be used to quantify the chitosan content in the nanoparticles.[53] As shown in Figure 6, increasing the amount of the chitosan decreased the absorbance due to the hydroxyl group stretch bonds of chitosan since these were used to form chemical bonds to the NPs.

The covalent conjugation of the chitosan molecules onto the PLGA molecules through the formation of an amide bond was confirmed by observing the bands around 1746 and 1510 cm^{-1}, corresponding to the amide I and II bands respectively. These amide I and II bands were not observed when higher amounts of chitosan were adsorbed onto the particles. The further addition of chitosan decreased the absorbance in this region, indicating the absorption of more chitosan molecules onto the surface of the PLGA particles.

3.4 Laboratory cytotoxicity of chitosan-modified PLGA NPs

The laboratory cytotoxicity of the synthesized samples in contact with the PC12 cell line – an important neural cell model – was assessed by the MTT assay. The PC12 line is a neuroendocrine cell model that is also useful as a model system for neuronal differentiation. Here, the chitosan-modified PLGA nanoparticles were tested at different dosages. As shown in Figure 7(a), for the test after 1 d and at lower dosages (2·5 and 25 µg/ml), none of the samples was toxic. For the dosage of 100 µg/ml, the cell viability of the samples containing lower amounts of chitosan was lower. This could indicate that using higher amounts of chitosan could further enhance the cell response. As shown in Figure 7(b), the test after 3 d also indicated no toxic effects for the 2·5 µg/ml dosage, or for 0·1% and 0·08% chitosan for any dosages. However, the 0·01 and 0·06% chitosan in the 25 and 100 µg/ml dosages showed the lowest cell viability when compared to the other samples. The outcomes showed that a dosage of 2·5 µg/ml was not toxic for any samples.

3.5 Blood-brain barrier transport study

The distribution of different chitosan surface-modified PLGA NPs in the brain was quantitatively compared using coumarin-6 loaded into the particles as a fluorescent marker (see Figure 8). All the samples showed an initial concentration increase during the first

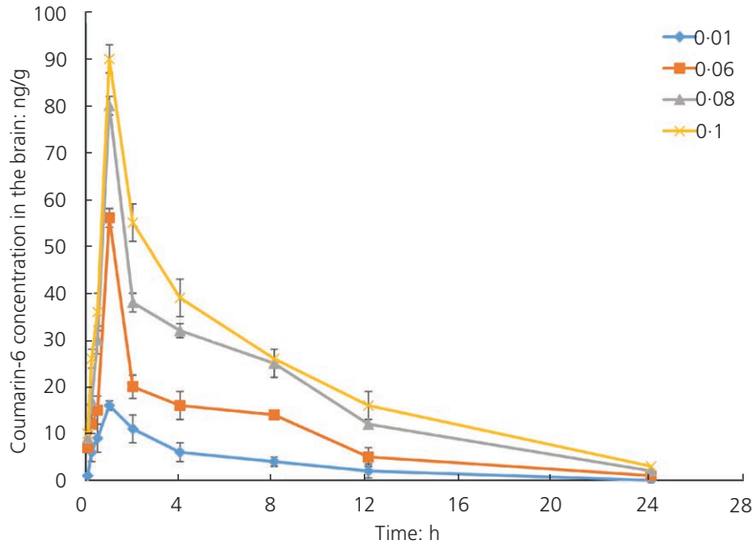

Figure 8. Coumarin-6 concentrations (ng/g) in mice brains after the injection of a 30 µg/kg dose of PLGA nanoparticles modified with different chitosan concentrations for different time intervals. The chitosan concentrations (% w/v) are shown with different line colors: 0·01 (blue), 0·06 (orange), 0·08 (gray) and 0·1 (yellow)

hour of the post-injection. However, the samples containing a higher amount of chitosan on the surfaces showed a meaningful increase in the brain accumulation, ranging from 16·3 to 89·7 ng/g. In addition, all the samples exhibited similar trends in their uptake profiles, suggesting that the positive charge of the chitosan molecules could play an important role in the transport of PLGA NPs across the blood-brain barrier. In other words, the proposed surface modification played a critical role in the brain distribution of PLGA NPs and improved its ability to serve as a carrier for the delivery of drugs to the brain. The presence of more chitosan ligands on the surfaces might increase the chance for PLGA NPs to interact with the blood-brain barrier, thereby leading to enhanced brain transport efficiency.

4. Conclusion

In conclusion, chitosan has been successfully used as an effective surface modifier for the preparation of PLGA NPs with desired characteristics. The smallest particles, in the range of nanometers, were obtained by increasing the amounts of chitosan. The authors previously found that PVA-emulsified NPs may be toxic, but the use of TPGS in this study confirmed that the residual TPGS molecules remaining on the surface did not need to be removed by washing because the TPGS and chitosan molecules were both efficacious in the treatment of brain diseases. The surface modification of NPs can therefore be a useful strategy for achieving the controlled and targeted release of active agents over an extended period.

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