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Short Communication

Preparation, characterisation and anti-tumour activity of *Ganoderma lucidum* polysaccharide nanoparticles

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Abstract

Objectives The aim was to prepare novel *Ganoderma lucidum* polysaccharide nanoparticles and to evaluate the physicochemical properties and anti-tumour activity in in-vitro cytotoxicity studies using HepG2, HeLa and A549 cancer cell lines, and growth promotion effects on mouse spleen cells.

Methods Chitosan nanoparticles loaded with *G. lucidum* polysaccharide were prepared using the ion-revulsion method. The diameter distribution of the particles and the surface charge were measured using a zetasizer analyser. The entrapment efficiency and drug loading capacity were examined by the diethylaminoethanol weak anion exchange method. The cytotoxic effects of nanoparticles on tumour cells and the growth promotion effects on mouse spleen cells were tested using the MTT assay.

Key findings Nanoparticles loaded with *G. lucidum* polysaccharide at 6 μg/ml and chitosan/sodium tripolyphosphate (mass) ratio of 5.5 had significantly greater cytotoxic effects on tumour cells and growth promotion effects on mouse spleen cells than empty nanoparticles.

Conclusions *G. lucidum* polysaccharide nanoparticles showed significant anti-tumour efficacy, having both cytotoxic effects on tumour cells and growth promotion effects on spleen cells, making it a promising candidate in the clinical setting.

Keywords chitosan; cytotoxicity; *Ganoderma lucidum* polysaccharide; nanoparticle; immune stimulation

Introduction

Ganoderma lucidum (*Lentinus edodes*) has been reported to be a medicinal mushroom for the treatment or prevention of many diseases, including AIDS, hepatitis B and cancer.[1] *G. lucidum* polysaccharide (GLP, *Lentinan*), a form of bioactive β-glucan, which is extracted from *G. lucidum,* is one of efficacious ingredient groups of *G. lucidum*. The structure of GLP is shown in Figure 1.

It has been demonstrated that GLP can preserve the vitality and promote longevity of cancer patients by stimulating the immune response and inhibiting growth of several kinds of cancer cells.[2] The anti-tumour effects of *G. lucidum* have been demonstrated both *in vitro*[3] and *in vivo*.[4] Paterson[5] and Stanley *et al*. [6] demonstrated that *G. lucidum* inhibited cell proliferation, induced apoptosis and suppressed cell migration in a highly invasive human prostate cancer cell line (PC-3). Zhu *et al*.[4] demonstrated the anti-tumour effects of *G. lucidum* polysaccharide *in vivo* using a mouse model. The underlying mechanisms for the treatment of cancer involve a direct cytotoxic effect of GLP on cancer cells and a cell-mediated immune response induced by this agent.

GLP is usually diluted in saline for intravenous administration; however, as *G. lucidum* is edible, it has also been administered orally in pharmacology studies.[7–9] However, there are some disadvantages associated with this route, such as low bioavailability and poor stability. Therefore, several novel drug delivery systems such as liposomes and microspheres[9–12] have been developed, with the aim of increasing the bioavailability and decreasing toxicity of GLP. *G. lucidum* liposomes prepared by Babincova *et al*.[9] and de la Fuente *et al*.[10] were shown to block the development of gastrointestinal tumours. Polysaccharide microcapsules and microspheres have also been made for the treatment of cancer.[11–13] Compared with

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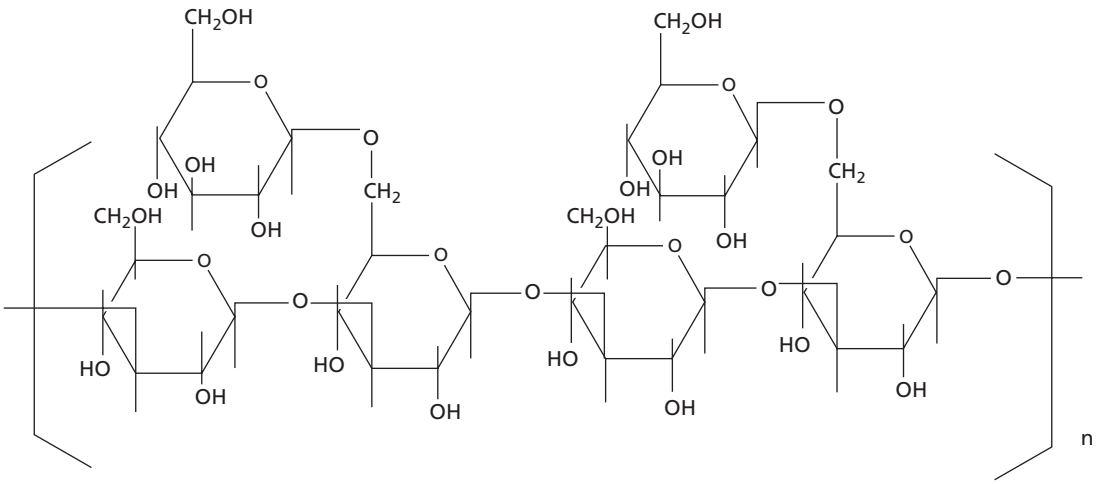

Figure 1 Structure of *Ganoderma lucidum* polysaccharide

conventional formulations, nanoparticles could escape endocytosis by cells and then circulate freely in the blood, making them suitable for targeted drug delivery. Thus, it is of great interest to develop GLP nanoparticles for applications in cancer therapy.

In the present study, we prepared novel chitosan nanoparticles loaded with GLP. The nanoparticles showed significant cytotoxic effects in tumour cells and promoted growth of spleen cells, making these nanoparticles a promising candidate in the clinical setting.

Materials and Methods

Drugs and reagents

Chitosan (85% deacetylation, MW 10^5 Da) was purchased from Yuhuan Oceanic Biochemistry (Taizhou, China). Sodium tripolyphosphate (TPP) was obtained from Shanghai Chemical Reagent Company of Chinese Medicine (Shanghai, China). GLP was kindly provided by the Institute of Chemical Biology and Pharmaceutical Chemistry, Zhejiang University. All other reagents were of analytical grade and were supplied by Huadong Medical (Shanghai, China).

Animals

Male ICR mice (5–7 weeks old; 25–30 g) were supplied by Zhejiang University Experimental Animal Center, China. Animals were maintained under constant conditions (temperature 25 ± 1°C) and had free access to a standard diet and drinking water. The animal experiments were approved by the ethical committee of Zhejiang University, and experimental procedures were in accordance with the Zhejiang University guidelines for the welfare of experimental animals.

Preparation of mouse spleen cells

Spleen cells were harvested as reported previously.[14] Spleen cells were harvested in RPMI 1640 complete medium supplemented with 10% fetal bovine serum, 100 units/ml streptomycin and 100 units/ml penicillin, and centrifuged at 1000 rpm for 10 min. The cell pellets were then resuspended and maintained in 2 ml RPMI 1640 complete medium. All procedures were performed under aseptic conditions.

Preparation of chitosan-GLP nanoparticles

Chitosan solution (2.5 mg/ml; 100 ml) was prepared by dissolving the polymer in 1% (v/v) acetic acid aqueous solution for 1 h under magnetic stirring; 10 ml GLP solution was then added. The pH of the solution was adjusted to 5.0 using 1 mol/l NaOH. The chitosan solution was then stirred for 1 h at room temperature. Finally, counterion TPP was dissolved in pure water to prepare a 1 mg/ml solution, which was added to the chitosan-GLP solution under mild magnetic stirring. The mixture was then shaken gently at 70 rpm for 1 h to form the chitosan-GLP nanoparticles. The nanoparticle solution was centrifuged at 22 000 rpm at 4ºC for 30 mins. After discarding the supernatant, the nanoparticles at the bottom were collected, washed with water and finally lyophilised.

Morphological characterisation of nanoparticles

The morphological examination of nanoparticles was performed by transmission electron microscopy. The nanoparticles were stained with 2% (w/v) phosphotungstic acid aqueous solution for 10 s, immobilised on copper grids with Formvard and dried overnight before microscopy.

The particle size and surface charge (represented by the surface zeta potential) were determined by laser diffraction spectrometry (Malvern Zetasizer 3000HS, Malvern, UK).

Determination of entrapment efficiency of GLP and loading capacity of nanoparticles

The quantity of GLP entrapped in the nanoparticles was calculated by the diethylaminoethanol (DEAE) weak anion exchange method.[15–17] The entrapment efficiency of the nanoparticles was calculated as: entrapment efficiency (%) = (total GLP − free GLP)/total GLP (mg). GLP loading capacity was calculated from: loading capacity (%) = (total GLP − free GLP)/weight of nanoparticles.

MTT assay

The cytotoxicity of GLP-loaded nanoparticles and free GLP was compared on the test cells (HepG2, HeLa and A549 cell lines) using the MTT method.[18] Briefly, the tumour cells were seeded at a density of 5×10^3 cells/well in 180 μl growth medium in 96-well plates and incubated for 24 h. Mouse spleen cells were seeded directly at a density of 5×10^3 cells/well in 180 μl growth medium in 96-well plates without incubation for 24 h. Then 20 μl of the different GLP preparations (free GLP, blank nanoparticles and GLP-loaded chitosan nanoparticles) was added to each well and the final volume adjusted to 200 μl with fresh serum-free DMEM. Cells were incubated with drug at 37ºC in a humidified 5% CO_2 atmosphere for 48 h. Then, 20 μl MTT solution (5 mg/ml) was added to each well and the OD_{595} values measured according to the manufacturer's instructions. All experiments were carried out in triplicate to ascertain the reproducibility. Cytotoxicity was calculated as: cytotoxicity (%) = [OD_{595} (control) − OD_{595} (sample)/OD_{595} (control)] × 100. The growth promotion in spleen cells was calculated as: growth promotion ratio (%) = [OD_{595} (sample) − OD_{595} (control) /OD_{595} (control)] × 100.

Statistical analysis

Data are expressed as means ± SD. Statistical analysis was performed using the Kruskal–Wallis test or Dunn's test. Differences were considered significant at $P < 0.05$.

Results

Formation and characterisation of nanoparticles

Chitosan nanoparticles loaded with GLP were successfully prepared by the ionic cross-linking of chitosan with TPP. As shown in Figure 2, the GLP-loaded nanoparticles maintained a round shape with almost homogeneous structure; they were 217 ± 6 nm in diameter. Figure 3 shows the effects of chitosan/TPP (mass) ratio on particle size and zeta potential of GLP-loaded nanoparticles. The particle size decreased as the chitosan/TPP ratio increased from 4 : 1 to 6.25 : 1, while the zeta potential increased. The entrapment efficiency and drug-loading capacities were calculated for the nano-preparations of different chitosan/TPP mass. No significant differences in the particle size (nm), entrapment efficiency or drug-loading capacity were found among the different chitosan/TPP ratios. The entrapment efficiency was 25.01 ± 0.95%, and the loading efficiency was 41.88 ± 0.89 % (Table 1), which is similar to reported values.[19]

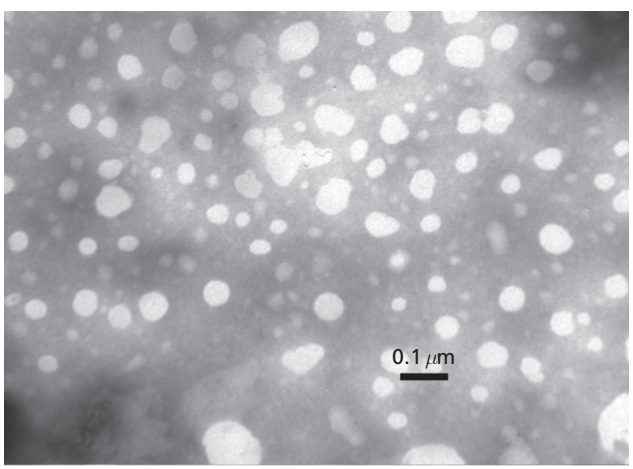

Figure 2 Transmission electron micrograph of chitosan nanoparticles loaded with *Ganoderma lucidum* polysaccharide

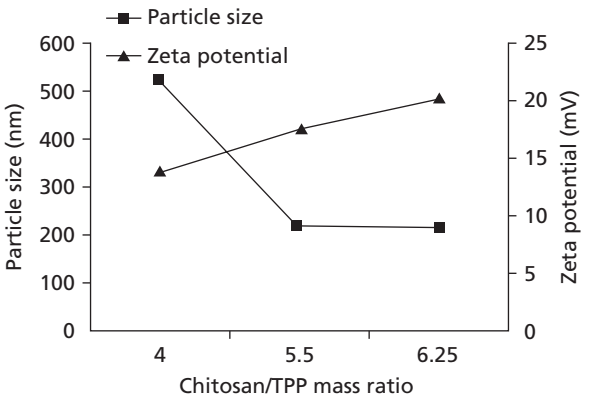

Figure 3 Particle sizes and zeta potential of GLP-loaded chitosan nanoparticles at different chitosan/TPP mass ratios.

Cytotoxicity of GLP-nanoparticles on tumour cells

The MTT assay was used to determine the cytotoxicity of GLP-loaded nanoparticles on tumour cells compared with a GLP solution and the empty nanoparticles. Figure 4 shows the in-vitro cytotoxicity of the chitosan-GLP nanoparticles (chitosan/TPP ratio 5.5) in three cell lines (HeLa, HepG2 and A549 cells). The chitosan-GLP nanoparticles had a higher inhibition ratio than free GLP solutions in the tested cells, especially when the GLP concentration reached 6 μg/ml ($P < 0.05$). The inhibition ratio of chitosan-GLP nanoparticles was dose dependent. The highest inhibition ratio was observed in the three cell lines at the GLP concentration of 6 μg/ml and was 41.6%, 37% and 45.3% for HepG2, HeLa and A549 cell lines, respectively.

Effects on growth promotion in mouse spleen cells

Besides the anti-tumour activities, GLP was also found to have immunomodulatory activities,[20] which involved the modulation of many components of the immune system such as antigen-presenting cells, natural killer cells and T and B lymphocytes.[2] Here, we tested the effects of GLP-loaded nanoparticles on the proliferation of mouse spleen cells compared with free GLP. As shown in Figure 5, the effects of chitosan-GLP nanoparticle

Table 1 Physiochemical properties of chitosan-GLP nanoparticles prepared with different chitosan/TPP mass ratios

Chitosan/TPP mass ratio	Particle size (nm)	Entrapment efficiency (%)	Drug-loading capacity (%)
5	227 ± 9	26.10 ± 1.4	42.90 ± 2.1
5.5	217 ± 6	24.70 ± 0.3	41.50 ± 1.7
6.25	205 ± 14	24.30 ± 0.9	41.25 ± 1.2

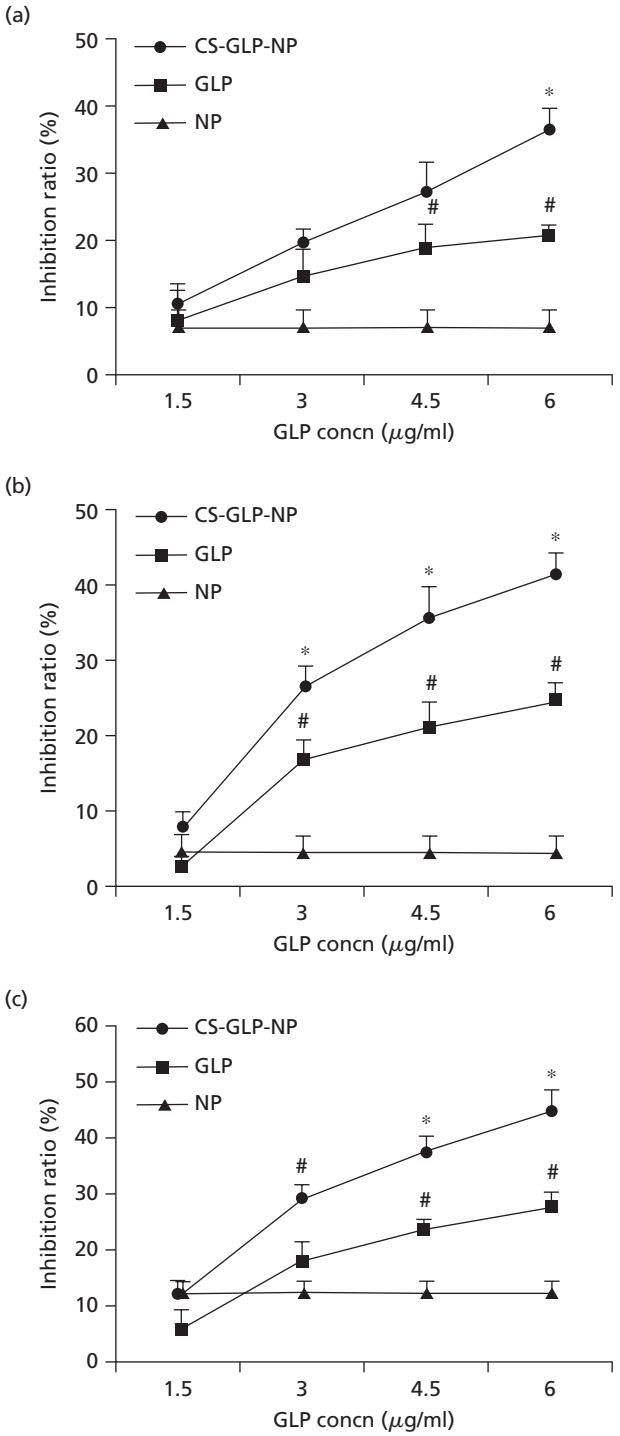

Figure 4 Inhibition ratios of GLP preparations in (a) HeLa cells, (b) HepG2 cells and (c) A549 cells. CS-GLP-NP, chitosan-GLP nanoparticles; GLP, GLP aqueous solution; NP, blank nanoparticles. Graphs show means ± SD ($n = 3$). $^{*}P < 0.05$, comparing all formulations; $^{\#}P < 0.05$ vs control group. (The comparison of the formulation type (NP, GLP, CS-GLP-NP) on the y variable at each concentration of GLP was performed using the Kruskal–Wallis test. Individual differences between the formulations were evaluated using Dunn's test.)

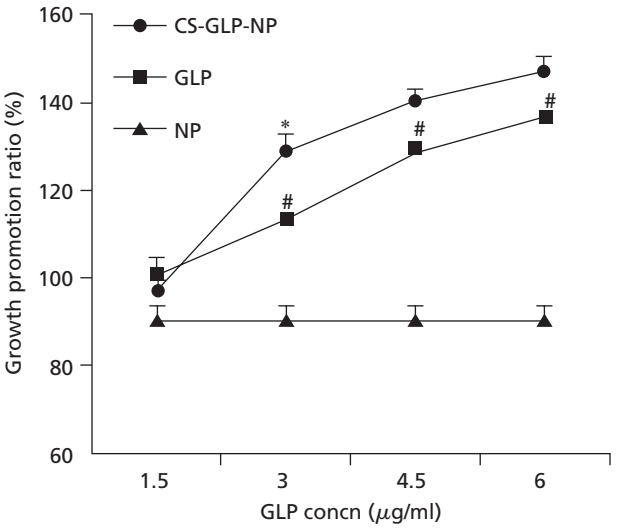

Figure 5 Growth promotion effect of GLP preparations on mouse spleen cells. CS-GLP-NP, chitosan-GLP nanoparticles; GLP, free GLP solution; NP, blank nanoparticles. Graph shows means ± SD ($n = 3$). $^{*}P < 0.05$ comparing all formulations; $^{\#}P < 0.05$ vs control group. (The comparison of the formulation type (NP, GLP, CS-GLP-NP) on the y variable at each concentration of GLP was performed using the Kruskal–Wallis test. Individual differences between the formulations were evaluated using Dunn's test.)

and free GLP on cell proliferation were dose dependent. The chitosan-GLP nanoparticles greatly increased the proliferation of spleen cells and showed a higher promotion ratio than free GLP, although the difference was not significant.

Discussion

In the present study, we prepared GLP-loaded nanoparticles using the ionic cross-linking method. Chitosan, a biocompatible material, was linked with counterion TPP to form an ionotropic gelatin. Nanoparticles prepared by this method are biocompatible and show low toxicity, as required for intravenous administration.[21–23] The high molecular weight and hydrophilicity of GLP make it difficult to encapsulate in solid particles.[24] Here, we successfully prepared GLP-loaded nanoparticles by the ionic cross-linking method. To the best of our knowledge, this kind of nanoparticle has not been reported previously. GLP-loaded chitosan nanoparticles formed instantaneously when the polyanionic TPP was added to the chitosan solution, which is in agreement with a previous report.[25]

The particle size of the nanoparticles increased with the increasing amount of TPP. However, the stability of nanoparticles decreased at the same time, especially when the chitosan/TPP ratio reached 7.5. Both particle size and stability of nanoparticles influence growth promotion effects on mouse spleen cells and anti-tumour effects on cancer cells.[26–30] We carefully adjusted the TPP concentration in the preparation of nanoparticles to obtain a balance between nanoparticle size and

stability. The chitosan/TPP ratios of 4, 5, 5.5 and 6.25 were selected for the particle size (nm) and surface charge study. Nanoparticles with a chitosan/TPP ratio of 4 had a particle size of 500–600 nm and were unstable; these were therefore not tested in the MTT assay. Smaller nanoparticles are easily taken up by cancer cells and suppress the growth and invasive behaviour of cancer cells.[31,32] Thus, the entrapment ratio and drug-loading ratio of the other three groups, with particle sizes of around 200 nm, were tested and showed no significant differences. Finally, by observing the particle shape and measuring stability, the optimal particle shape and structure were found in nanoparticles with a chitosan/TPP ratio of 5.5, which were therefore chosen for the in-vitro tumour inhibition and spleen cell proliferation tests.

Compared with the GLP solution, the GLP nanoparticles showed a higher anti-tumour efficiency, which involved a direct cytotoxic effect on cancer cells, and greater proliferation effects on lymphocytes from mouse spleen. The nanoparticles themselves may have some cytotoxicity, and it is possible that the enhancement in cytotoxicity of the GLP-loaded nanoparticles comes from the nanoparticle. We therefore included a control of free GLP mixed with empty nanoparticles for comparison. The GLP nanoparticles showed a higher anti-tumour efficiency than the free GLP mixed with empty nanoparticles, demonstrating that the GLP nanoparticles are the better formulation.

The concentration of GLP used for the cytotoxicity study was 1.5–6 μg/ml; the in-vitro cytotoxic effect of GLP did not increase at concentrations higher than 6 μg/ml, as also shown by Cao and Lin[33] and in experiments conducted in our laboratory (data not shown).

It was assumed that the anti-tumour effects of GLP *in vivo* are not merely induced by the cytotoxic effect of GLP on tumour cells. The immune stimulation of GLP on lymphocytes also plays an important role in the treatment of cancer *in vivo*.[27,30] Specifically, GLP stimulates the immune system, resulting in the production of cytokines and activating the anti-cancer activities of immune cells.[22] Here, we assumed that with a suitable particle of about 200 nm and ideal stability of the nanoparticles produced, these GLP-loaded chitosan nanoparticles were more readily taken up by tumour cells (by endocytosis) than the GLP solution, followed by release of the GLP from the nanoparticles to kill the cancer cells.

Taking together the increased cytotoxic effects on tumour cells and immune-stimulating effects on spleen cells, along with the low cytotoxicity of biocompatible materials used, these GLP-loaded nanoparticles could be effective in cancer therapy. Further preclinical study is warranted to investigate the potential of GLP-loaded nanoparticles for cancer therapy.

Conclusions

GLP nanoparticles were prepared and characterised. Physical properties were optimal at a chitosan/TPP mass ratio of 5.5. The GLP nanoparticles showed greater tumour inhibition and growth promotion effect than free GLP solution, making these a promising candidate for cancer treatment in the clinical setting.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

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