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Enhanced delivery of baicalein using cinnamaldehyde cross-linked chitosan nanoparticle inducing apoptosis

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ABSTRACT

The chitosan (CS) nanoparticles, baicalein loaded chitosan nanoparticles were prepared by crosslinking method in a W/O emulsion system, using cinnamaldehyde as crosslinking agent. The FT-IR result showed the binding of anticancer compound baicalein to the nanoparticles. The TEM analysis revealed that the particles are spherical in nature. Zeta potential revealed negative charge of the particles. Ultraviolet spectrum analysis described that higher loading efficiency and encapsulation efficiency as 9.1% and 97.2%, respectively. In vitro baicalein release profile demonstrated the delivery of baicalein from the CS nanoparticles is a two stage process. RT-PCR and cell culture was carried out accordingly.

1. Introduction

The potential use of polymeric nanoparticles as drug carriers/drug delivery vehicles has led to the development of many different colloidal delivery vehicles. The main advantages of this kind of systems lie in their capacity to cross biological barriers, to protect macromolecules such as peptides, proteins, oligonucleotides, and genes from degradation in biological media, and to deliver drugs or macromolecules to a target site with following controlled release [1]. Chitosan is a functional linear polymer and can be derived by partial deacetylation of chitin. It is the most abundant natural polysaccharide on the earth after cellulose, which can be extracted from exoskeleton of marine crustaceans such as crabs, lobsters, shrimps and krill. Chitosan is a copolymer consisting of 2-amino-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-glucose units linked with beta-(1→4) bonds [2,3]. Chitosan nanoparticles are good drug carriers because of their good biocompatibility, biodegradability and readily modifiable properties [4]. A good drug carrier can overcome the disadvantages of commonly used drugs such as poor stability, water insolubility, low selectivity and high toxicity. Chitosan nanoparticles are drug carriers with wide development potential and has the advantage of slow/controlled drug release, which improves drug solubility, stability, enhances efficacy, and reduces toxicity. Because of their small size, they are capable of passing through biological barriers in vivo (such as the blood–brain barrier) and delivering drugs to the lesion site to enhance efficacy [5]. Chitosan of suitable molecular weight are cleared by the kidney in vivo, while that of molecular weight can be degraded into fragments suitable for renal clearance [6]. The poor physical/mechanical properties and increased swelling in aqueous system limits the practical application of chitosan in molecular imprinted polymer preparation. One way to overcome this limitation is chemical cross-linking. Bifunctional reagents such as glutaraldehyde are frequently used as cross-linking agents [7]. Glutaraldehyde cross-linked with hyaluronic acid hydrogel films are having low stability when compare to those treated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, poly(ethylene glycol) diglycidyl ether, and divinyl sulfone. Interestingly, the glutaraldehyde-crosslinked films show a large initial increase in volume before a reduction in swelling occurs. This may be the possible reason for crosslink scission and the swelling can mainly be attributed to main-chain degradation. Thus, the reaction has been accomplished under acidic, neutral, and alkaline conditions using carbodiimides, hydrazides, aldehydes, sulfides, and polyfunctional epoxides [8]. Glutaraldehyde is classified as toxic substance; repeated exposure causes irritation of eyes, nose, throat or skin resulting in dermatitis and asthma [9]. So, cinnamaldehyde is used as crosslinking agent instead of glutaraldehyde. Cinnamaldehyde can inhibit the proliferation of several human cancer cell lines including those established from breast, ovarian, lung, and colon carcinomas and leukaemia's [10]. Baicalein (5,6,7-trihydroxy-2-phenyl-4H-1-benzo-pyran-4-one) is a natural flavonoid constituent derived from the root of *Scutellaria baicalensis*

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Georgi. It protects from a wide spectrum of oxidative injuries [11,12] and is able to inhibit the growth and multiplication of different cancer cells [13–15]. Recent investigations have shown that *S. baicalensis* alone, or in combination with other herbs, can inhibit cancer cell growth or induce apoptosis in breast, hepatocellular, pancreatic, prostatic and urothelial carcinoma cell lines [16–19]. The currently marketed oral preparations including tablets and capsules have poor solubility thus reduced dissolution and bioavailability [20]. Baicalein mediates MDA-MB-231 invasive properties by suppressing the activation and expression of MMP-2 and MMP-9, which are very important factor for breast cancer cells. MAPK/MMP signaling in MDA-MB-231 cells contributes to the underlying mechanism of invasive potential, which suggests that MAPK signaling is a potential hallmark and a therapeutic target in highly invasive breast cancer cells [21].

In this study we made a possible mechanism to deliver the baicalein through chitosan nanoparticles, which are cross-linked with cinnamaldehyde by W/O emulsion crosslinking method to MCF7 breast cancer cells. High encapsulation and controlled release of CS nanoparticles were observed in this crosslinking method. The morphology of CS nanoparticles was confirmed by TEM, Zeta potential showed the surface charge of the particles. The ultraviolet spectroscopy revealed the baicalein loading efficiency and *in vitro* baicalein release. The cytotoxicity of nanoparticles was evaluated by MTT assay, the RT-PCR exhibited the expression of gene and Hoechst 33258 staining of MCF7 exposed the apoptotic activity of the synthesized baicalein loaded nanoparticles.

2. Experimental

2.1. Chemicals and reagents

Chitosan (CS) (low molecular weight), baicalein (98%), Hoechst 33258, MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide), were purchased from Sigma India. Petroleum ether, sodium hydroxide, liquid paraffin and *n*-butanol were purchased from Himedia. All other chemicals were reagent grade and were used without further purification.

2.2. Preparation of baicalein loaded nanoparticles

Baicalein was dissolved in ethanol to make a final concentration of about 3 mg/mL, 6 mg/mL and 9 mg/mL. This was poured into a fine dispersion of 40 mL of liquid paraffin containing 2 mL of *n*-butanol and stirred for 15 min. About 100 mg of chitosan was dissolved in 10 mL of 1% acetic acid, add 0.5 mL of cinnamaldehyde and stir for 10 min. Now the aqueous phase was poured into oil phase and stirred for 3 h, pH was adjusted with the help sodium hydroxide. Finally the whole mixture was washed with ethyl acetate and finally with double distilled water repeatedly and centrifuged at 12,000 rpm for 35 min. The resulting processed nanoparticles were lyophilized. The nanoparticles prepared without adding baicalein was used as control. The size of nanoparticles was determined by adjusting pH of the system and the nanoparticles were obtained by crosslinking of cinnamaldehyde by "Schiff base".

2.3. Measurement of size, morphology and surface charge of nanoparticles

The particle size distribution and zeta potential were measured by photon correlation spectroscopy (PCS) with a zeta potential analyzer add-on unit (Nicomp Zetasizer 380ZLS, Urbana, IL, USA). Samples were diluted to the desired concentration and two 5 min cycles were run for each sample with water prior to the measurements. Intensity-weighted size distributions are reported. For zeta potential measurements, three 30 s cycles were run for each sample. The morphology was examined by transmission electron microscopy (TEM) (Hitachi 7000H, Tokyo, Japan). The nanoparticles solutions were sonicated for 1 min to produce better particle dispersion and to prevent nanoparticle agglomeration on the copper grid. After this, drop of the nanoparticle solution was spread onto a carbon-coated copper grid, which was then dried at room temperature. The sample was then examined and photographed under the microscope.

2.4. FT-IR characterization of chitosan nanoparticles

Fourier transform infrared (FT-IR) spectroscopy of CS, blank CS nanoparticles and baicalein loaded nanoparticles were performed by using Nicolet 5700 instrument (Nicolet Instrument, Thermo Company, USA) with KBr pellet method. Each KBr disk was scanned over a wave number region of 500–4000 cm^{-1} with the resolution of 4.0×10^8 cm.

2.5. The baicalein loading efficiency and encapsulation efficiency

Baicalein 3 mg loaded CS nanoparticles was dispersed into 6 mL of phosphate buffer solution (PBS) and centrifuged at 12,000 rpm for 30 min. The supernatant was collected to measure the ultraviolet absorption at 272 nm. The loading efficiency and encapsulation efficiency of baicalein loaded CS nanoparticles were calculated as follows [22]:

$$\text{Loading efficiency} = \frac{W_0}{W} \times 100\%$$

$$\text{Encapsulation efficiency} = \frac{W_0}{W_1} \times 100\%$$

where W_0 is the weight of baicalein enveloped in the CS nanoparticles, W is the weight of CS nanoparticles, and W_1 is the amount of baicalein added in the system.

2.6. In vitro drug release studies

Baicalein was dissolved in ethanol to obtain the final concentration of the solution 1×10^{-6} mol/L, 5×10^{-6} mol/L, 10×10^{-6} mol/L, 15×10^{-6} mol/L and 20×10^{-6} mol/L, then ultraviolet absorption at 272 nm was measured, respectively. The regression equations can be worked out by the concentration–absorbance standard curve. The CS nanoparticles loaded with 10 mg/mL baicalein were dispersed in 5 mL PBS (pH = 7.4), putting in dialysis tubing (MW3500, MD24, USA), and incubated in 200 mL PBS (pH = 7.4) at 37 °C under slight shaking. At regular intervals, 5 mL of aliquots was removed and measured the ultraviolet absorption at 272 nm, and then isometric fresh PBS was added to maintain the constant volume. The accumulative drug release–time curve was obtained according to the standard curve.

2.7. In vitro cytotoxicity

The cytotoxicity of CS nanoparticles was evaluated by MTT (3-(4,5-dimethylthiahiazol-2-yl)-2,5-diphenyl tetrazolium) assay. MCF 7 breast cancer cells were seeded onto a 96-well plate with a density of 2×10^4 cells/mL. The prepared blank CS nanoparticles and baicalein loaded CS nanoparticles were dispersed in PBS (pH = 7.4) to obtain a final concentration of 15 μg/mL, 30 μg/mL, 45 μg/mL and 60 μg/mL, and then added to each well respectively. Baicalein was added at the same concentration using as positive control. Cells were incubated in a CO_2 incubator at 37 °C for 48 h. MTT solution was added 100 μL/well, followed by incubation for 4 h. The supernatant was removed, and dissolved with 100 μl of