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In vitro comparative studies of Zein nanoparticles and composite Chitosan thermogels based injectable formulation of Doxorubicin

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ABSTRACT

Surgical intervention of the solid tumours is the most preferred cancer treatment strategy in the current scenario. But, the main issue is that there is always a probability of resurrection of tumor due to the fact that not all cancer cells can be removed completely. In order to overcome this, controlled delivery of the therapeutic agents into the tumor region after the surgical removal of tumor seems to be a viable option. We developed a composite injectable Chitosan gel (DZ-CGs) comprising of Doxorubicin loaded Zein nanoparticles (DOX-SC ZNPs) which flows (in their sol state) and can take up the exact shape of the void (created by the surgical removal of the tumor) and becomes gel (at physiological temperature). DOX-SC ZNPs were synthesized by anti-solvent nano-precipitation method. The size and zeta potential of DOX-SC ZNPs were found to be 120 ± 16 nm and −26.97 mV respectively. *In vitro* drug release profiles of DZ-CGs were found to be more controlled when compared to DOX-SC ZNPs. *In vitro* cyto-toxicity studies of DOX-SC ZNPs and DZ-CGs were compared on human breast cancer cell lines using Transwell insert method and found that composite DZ-CGs were more effective in killing cancer cells when compared to DOX-SC ZNPs.

1. Introduction

Cancer is one of the leading causes of death around the world. Though there are several means of treating a solid tumor, even today the most preferred and successful treatment is by means of surgical removal of the solid tumor. But even this mode of treatment is not without pitfalls. The main drawback is that not all cancerous cells can be completely removed after a surgery. There is always a probability of resurrection of tumor (from the left over cancerous cells), which is a big concern for the medical community [1–3]. In order to kill the left over cells and prevent the further growth of tumor, chemotherapeutic drugs are delivered either systemically or locally. The problem with systemic delivery of chemo drugs is that it causes serious toxicity issues to healthy tissues. In order to reduce the toxicity problem of chemo-drugs, nanoparticles based drug delivery systems which could encapsulate the drug and deliver them exactly at the target region in controlled manner are currently being researched extensively [4,5]. But, after this many years of intense research in nanoparticles based drug delivery systems, it was observed that these nano-particulate system has not lived up to its expectations as a targeted drug delivery systems. It was found that nearly 95% of the systemically administered drug loaded nano-carriers did not reach the tumor region, which is really a matter of concern [6–8].

Local delivery of the therapeutic agents into the tumor region has many advantages over the systemic delivery of these drugs. They are as follows: Firstly, therapeutic effectiveness can be achieved using lower amount of drug. Secondly, systemic side-effects of chemo-drugs can be dramatically minimised. Thirdly, drug could be tuned to release in a controlled manner and ensure sufficient diffusion and uptake into cancer cells over many cycles of tumor cell division [9–11]. Thus, to overcome these difficulties local drug delivery systems which can deliver the drug in a controlled manner is the need of the hour. Presently, Polymer based local drug delivery systems are widely studied controlled drug delivery systems [12–15].

Injectable thermosensitive gel or *in situ* thermogels are one such local drug delivery system which are solution at room temperature and forms a gel at physiological temperature inside the body, which can then act as a local drug delivery depot. As these gels are in sol

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(liquid) state at room temperature, they can be injected into the target site easily. Moreover, as these gels flow (in their sol state) and then gel in response to temperature, they can take up the exact shape of the void (created by the surgical removal of the tumor) and fill them. Some of commonly researched injectable thermogels are pluronics, PEG based co-polymeric systems, peptide based gels, PNIPAM based gels, Chitosan gels, Cellulose derivatives based gels etc. [16–18].

Chitosan based thermogels are one of the most widely researched natural polymer based gels [19–21]. These are biodegradable gels and so do not require any additional surgery to remove them. But there are few issues associated with this Chitosan based thermogels [31] like, the uncontrolled drug release profile and non-uniform dispersion of the anticancer drugs due to their high hydrophobicity [22].

To overcome these drawbacks, we propose our hypothesis of nano composite in gel form where drug loaded nanoparticles are embedded inside a thermogel. Here the drug delivery is restricted by the nanoparticle as well as the hydrogel boundary. Eventually this composite gel system favours the uniform dispersion of drug inside the hydrogel as well as controlled drug release to the affected site [23]. Also the gel prevents the escape of drug loaded nanoparticles from the tumor region, causing reduced toxicity to the surrounding tissues. The proposed nano composite hydrogel can be used for dual drug loading, one in the hydrogel and the second in the nanoparticles embedded in the composite gel. A controlled drug delivery can be achieved using this method.

In this work, we synthesized Doxorubicin loaded Zein nanoparticles which were then embedded inside Chitosan thermogel to develop a composite nano-gelling system. Doxorubicin, anthracycline antitumor antibiotic is a potent anti-cancer drug that is currently used in wide gamut of cancer treatments [24]. Zein is an amphiphilic protein from maize (corn), which has GRAS (Generally regarded as safe) status from US FDA and is currently being explored as a controlled drug delivery carrier [25–28]. We synthesized Doxorubicin loaded Zein nanoparticles and optimized protein to drug ratio in order to get the maximum encapsulation efficiency. *In vitro* biocompatibility studies were carried out for Zein nanoparticles. Zein nanoparticles loaded composite injectable Chitosan gel was also synthesized. *In vitro* drug release studies were performed and compared between nanoparticles system and the composite system. *In vitro* cyto-toxicity studies of nanoparticles and the composite systems were compared on human breast cancer cell lines MCF-7 using transwell insert method.

2. Materials and methods

2.1. Materials

Chitosan (Medium Molecular weight having molecular weight of about 190–310 KDa, 75–85% Deacetylation), Zein from maize, Sodium caseinate from bovine milk, MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), were purchased from Sigma Aldrich, India. Ammonium Hydrogen Phosphate (AHP) purchased from Merck, India. Doxorubicin hydrochloride was procured from Cipla Limited. All other commercial reagents were of analytical grade and were used without any further purification.

2.2. Methods

2.2.1. Preparation of bare Zein nanoparticles (Bare-SC ZNPs)

Zein nanoparticles were prepared by anti-solvent nanoprecipitation method with slight modification [35]. About 1 ml of 2% Zein solution prepared in 80% ethanol was added dropwise to 3 ml of 2% Sodium Caseinate solution with constant stirring at 800 rpm to get stable Zein nanoparticles. The formed nanoparticles were collected by centrifugation at 15,000 rpm for 30 min. These Zein nanoparticles were then used for further characterizations. In a similar way, the bare Zein nanoparticles (without any stabilizer) were also prepared [25].

2.2.2. Preparation of doxorubicin loaded Zein nanoparticles (DOX-SC ZNPs)

DOX-SC ZNPs were prepared by anti-solvent nanoprecipitation method similar to that of bare SC ZNPs [34]. Briefly, 1 mg Doxorubicin was dissolved into 1 ml of the 2% Zein solution. The above solution was added dropwise into 3 ml of 2% Sodium Caseinate solution with stirring rate of 800 rpm, which resulted in the formation of stable nanoparticles dispersions. The formed nanoparticles were collected by centrifugation at 15,000 rpm for 30 min. Thus prepared Zein nanoparticles were redispersed and used for further characterizations.

2.2.3. Preparation of DOX-ZNPs loaded chitosan gel (DZ-CGs)

10 mg of prepared DOX-SC ZNPs were dispersed into 1 ml of 1.6% chitosan solution (having pH 5.6) and magnetically stirred in an ice bath. To this cooled chitosan solution, 20 μl AHP was added drop wise and the resulted solution (having pH 7.2) was incubated at 37 °C for 12 min to form composite injectable chitosan gelling system [30]. We used the protocol from Ref. [29] with slight modification where they had synthesized bare injectable Chitosan gel.

2.2.4. Characterization of DOX- SC ZNPs

2.2.4.1. Drug loading and entrapment efficiency. Zein to Doxorubicin ratio was varied and optimized to get the maximum encapsulation efficiency. The quantification of the drug Doxorubicin (λ_{max} = 480 nm) was done using UV-Vis Spectrophotometer (Shimadzu). The calibration curve for Doxorubicin (concentration: 3.125μg/ml-50 μg/ml) with the regression equation y = 20.375x-0.0083, with R^2 value of 0.9997 was obtained. In case of DOX-SC ZNPs, the supernatant present after the centrifugation of the formed nanoparticles was used to calculate entrapment efficiency. All other characterizations were performed on the formulation having the highest entrapment efficiency. Here the formula used was

$$\text{Encapsulation Efficiency } (\%) = \frac{I - S}{I} * 100$$

where,

I- Total amount of Doxorubicin taken initially for encapsulation (mg)

S- Amount of Doxorubicin present in the supernatant (mg)

$$\text{LoadingEfficiency}(\%) = \frac{\text{AmountofDoxorubicinencapsulated} * 100}{\text{TotalweightoftheZeinnanoparticles}}$$

2.2.4.2. Size, morphology and zeta potential analysis of DOX-SC ZNPs. The size distributions of the nanoparticles were characterized by dynamic light scattering and their surface charge by using a zeta analyzer (Brookhaven Instruments Corporation, USA). The size, shape and the surface morphology of the Zein nanoparticles were observed using FEG-SEM. In case of DOX-SC ZNPs, the formulation which has the highest entrapment efficiency was chosen for SEM analysis. The sample for the SEM analysis of Zein nanoparticles were prepared by drop-casting the sample on to one side of stub,