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In vitro and in vivo toxicity assessment of alginate/eudragit S 100-enclosed chitosan–calcium phosphate-loaded iron saturated bovine lactoferrin nanocapsules (Fe-bLf NCs)

Khoo Miew Leng[a], Soundararajan Vijayarathna[a], Subramanion L. Jothy[a], Sreenivasan Sasidharan[a,*], Jagat R. Kanwar[b,*]

[a] *Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, USM 11800, Pulau Pinang, Malaysia*
[b] *Nanomedicine-Laboratory of Immunology and Molecular Biomedical Research (LIMBR), School of Medicine (SoM), Faculty of Health, Deakin University, Waurn Ponds, Geelong, VIC 3216, Australia*

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ABSTRACT

Lactoferrin has been known to have antimicrobial properties. This research was conducted to investigate the toxicity of Alginate/EUDRAGIT® S 100-enclosed chitosan–calcium phosphate-loaded Fe-bLf nanocapsules (NCs) by *in vitro* and *in vivo* assays. Brine shrimp lethality assay showed that the LC_{50} value of NCs was more than 1 mg/mL which indicated that NCs was not toxic to Brine shrimp. However, the LC_{50} values for the positive control potassium dichromate at 24 h is 64.15 µg/mL, which was demostrated the toxic effect against the brine shrimp. MTT cytotoxicity assay also revealed that NCs was not toxic against non-cancerous Vero cell line with IC_{50} values of 536 µg/mL. Genotoxicity studies by comet assay on Vero cells revealed that NCs exerted no significant genotoxic at 100 µg/mL without tail or shorter comet tail. *Allium cepa* root assay carried out at 125, 250, 500 and 1000 µg/mL for 24 h revealed that the NCs was destitute of significant genotoxic effect under experimental conditions. The results show that there is no significant difference ($p > 0.05$) in mitotic index between the deionized water and NCs treated *Allium cepa* root tip cells. In conclusion, no toxicity was observed in NCs in this study. Therefore, nontoxic NCs has the good potential to develop as a therapeutic agent.

1. Introduction

Nanotechnology is a speedily rising area having potential applications in many fields. For the past few decades various formulations of nanoparticles have been used for drug delivery research to increase therapeutic benefit [1]. Up to now, nanoparticles (NPs) have been prepared from metal and non-metal, polymeric materials and bioceramics [2]. Due to their unique physicochemical and electrical properties, nano-sized materials have gained substantial attraction in the field of electronics, biotechnology, and aerospace engineering [2]. In the field of medicine NPs are being used as a novel delivery system for drugs, proteins, DNA, and monoclonal antibodies [3–5]. Key components in the nanoparticle preparation may help to enhance the therapeutic properties of nanoproducts. Alginate is a pH sensitive FDA approved polymer which protects drugs in the low acidic pH conditions inside the stomach and able to release drugs in the alkaline pH in the intestinal regions where all the absorption occurs [6,7]. Eudragit S 100 and chitosan polymers are mucoadhesive and able to stick to the intestinal walls and help in endocytosis and exocytosis through the intestine to the main blood streams for further distribution of drug molecules in the different parts of the body [8–10]. Nevertheless, many challenges must be overcome if the application of nanotechnology is to increase therapeutic benefit and yield improved therapies. Research has established that contact to these combustion derived ultrafine particles/nanoparticles is associated with a wide variety of effects [11]. In view of the potential applications of NPs in many fields and the rising worries of FDA about the toxic potential of nanoproducts, it is the need of the hour to study the toxicological effects of nanoproducts. In this study for the first time the toxicity of alginate-enclosed chitosan-calcium phosphate-loaded Fe-bovine lactoferrin nanocapsules (NCs) was reported by using brine shrimp lethality assay, *Allium cepa* assay, comet assay and cell cytotoxicity assay, especially in case of genotoxicity.

In vivo techniques includes *Allium cepa* assay and brine shrimp lethality assay [12] was used in this study. However, techniques to detect *in vitro* cytotoxicity involve the use of comet assay, and tissue culture [13] methods were also used in this study. The cytotoxicity of NCs was conducted using Vero cell, a non-cancerous cell to observe cytotoxicity of NCs in a cellular model. Subsequently, the comet assay

* Corresponding authors.
E-mail addresses: srisasidharan@yahoo.com (S. Sasidharan), jagat.kanwar@deakin.edu.au (J.R. Kanwar).

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was conducted to evaluate *in vitro* genotoxicity of NCs. Higher plants such as *Allium cepa* are good genetic models to recognise genotoxicity such as chromosome aberrations and changes in the mitotic cycle of NCs. Therefore, the current study utilized the root tips cells of *Allium cepa* for evaluating the genotoxic effects of NCs.

2. Materials and methods

2.1. Preparation of endotoxin-Free Fe-bLf

Endotoxin-free bovine lactoferrin (bLf) was prepared from Australian bovine milk [14]. Endotoxin was evaluated by using Genscript ToxinSensor™ (NJ, USA), Chromogenic Limulus Amebocyte Lysate Endotoxin Assay Kit (Genscript ToxinSensor). Ferum-bLf was produced from bLf based on the previously described method established in Deakin's laboratory, Australia. Following treatment with mild acidic solution (pH 2.6), bLf was dialyzed for a duration of 48 h in 0.1 M citric acid to remove bound metal ions and then saturated with ferric [Fe(III)] nonahydrate to form bLf- Fe^{3+} co-ordinate complexes to produce deep-red colored Fe-bLf. The Fe-bLf produced was loaded on to the chitosan–calcium phosphate NCs and coated with alginate.

2.2. Preparation of alginate/eudragit® S 100-enclosed chitosan–calcium phosphate-loaded Fe-bLf nanocapsules (NCs)

Alginate/EUDRAGIT® S 100-enclosed chitosan–calcium phosphate-loaded Fe-bLf nanocapsules (NCs) were produced by a combination of nanoprecipitation and ionic gelation methods [6,7]. Calcium phosphate was prepared from its constituents by adding disodium hydrogen orthophosphate (Na_2HPO_4) in a molar ratio of 4:1 to calcium chloride ($CaCl_2$) in a drop-wise manner while stirring continuously. The suspension was sonicated at 4 °C to ensure a white precipitate of calcium phosphate was obtained. The calcium phosphate (1% w/v) suspension produced was incubated for 24 h with 10% w/w Fe-bLf (from Section 3.2.1) with constant stirring at 4 °C, at a pH ≤ 8.0, the isoelectric pH of lactoferrin, to adsorb Fe-bLf on to the NCs. Following the electrostatic interaction of Fe-bLf on calcium phosphate (ceramic core), the suspension formed was centrifuged and washed several times to eliminate traces of unbound protein and then freeze-dried. 0.01% w/w chitosan solution in acetate buffer (pH 4) was added to calcium phosphate under constant stirring [7]. Then, 0.01% of cross linking agent, sodium tripolyphosphate (STPP) was added drop wise. Constant stirring at 6000 rpm (MSH-20D, Laboratory Instruments) for 12 h was performed to ensure that the nanoformulation attained the size of 200 ± 40 nm, followed by freeze drying to ensure spherical shape of the samples is acquired. These nano cores were then coated with alginate gel by using 2% w/v EUDRAGIT® S 100/alginate solution and calcium chloride, with 0.6% mass ratio of calcium alginate. Finally, the nanocarriers formed were washed and lyophilized. All these experiments were conducted at 4 °C as to protect the polymeric and protein constituents in the formulation.

2.2.1. Estimation of lactoferrin loaded onto AEC-CP-nanocarriers

Encapsulation efficacy of the nanoparticles was calculated using the ratio between bound and total protein added to the formulation. The formula used was: Encapsulation efficacy (%) = (total protein loaded-total unbound protein)/total protein loaded × 100.

2.2.2. Characterization of nanoparticles

Dynamic light scattering (DLS) was done using a zetasizer, after 500-fold dilution with autoclaved milliQ water to determine the average size of the prepared NCs following various steps of their preparation viz, calcium phosphate core, chitosan formulation and alginate enclosed NCs. Surface morphology of these particles was determined by scanning electron microscopy (SEM), at an accelerating voltage of 5–10 kV (Zeiss Supra).

2.3. Brine shrimp lethality assay

2.3.1. Hatching the shrimp

Brine shrimp eggs (*Artemia salina*) were hatched in a shallow rectangular basin filled with artificial sea water which was prepared by dissolving 38 g of sea salt in 1L of distilled water. The eggs were sprinkled into the rectangular basin. After an incubation for 24 h in a temperature-controlled room at 28 °C, under a continuous light regime. Subsequently, the larvae were attracted to one side of the container by a light source and collected with a pipette. Larvae were separated from the eggs by aliquoting them three times in a small beaker containing sea water [15].

2.3.2. Brine shrimp lethality test

The cytotoxicity of NCs was monitored by the brine shrimp lethality test [16]. NCs was dissolved in deionized water and diluted with artificial seawater. Two mililiters of seawater was pipetted in all the Bijoux bottles. Two fold serial dilution was conducted to obtain the concentration of NCs from 19.53 to 5000.00 μg/mL. Potassium dichromate was used as the positive control and was dissolved in sea water to obtain the concentration from 1.953 to 500.000 μg/mL. The negative control was filled with artificial salt water and deionized water only. Approximately 10–15 larvae were transferred into each Bijoux bottle and incubated for 24 h. Brine shrimp lethality test were done in a temperature-controlled room at 28 °C, under a continuous light regime. The larvae could be counted in the pipette tip against a lighted background. The number of dead larvae were counted in each bottle with the aid of a magnifying glass. The total number of larvae in each bottle was enumerated and recorded. The mean of the mortality percentage was plotted against the logarithm of concentrations. The concentration that could kill 50% of the larvae (LC_{50}) was determined from the graph.

2.3.3. Statistical analysis

Lethal concentration (LC_{50}) for *Artemia salina* was determined by Probit analysis using a Finney computer programme (Biostats 2009) [17]. Mortality percentage was corrected for the natural mortality found in the negative controls using Abbott's formula, $p = (p_i\text{-}C)/(1\text{-}C)$ where p_i represents the observed mortality rate and C denotes the natural mortality.

2.4. In vitro cellular model cytotoxicity assay

2.4.1. Cell culture

The Vero cell line used in this experiment was obtained from the Cell Culture Collection of the Tissue Culture Laboratory of the Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Penang. The Vero cell line was maintained as monolayer cultures in Dulbecco's Modified Eagle's Medium (DMEM) media with humified atmosphere in a 5% CO_2 incubator (Model-311, Thermo Electron Corporation, USA) at 37° C.

2.4.2. Cytotoxicity assay

In vitro cytotoxicity of NCs against Vero cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay by using a modified method [18]. Vero cells were grown in 25 cm^2 culture flask for two days in a CO_2 incubator at 37 °C in DMEM. Supernatant was discarded by using a pipette and cells attached on the surface of culture flask were washed twice with 1–2 mL of PBS (Gibco, Life Technologies, UK). Vero cells that attached on the culture flask were detached from the flask by adding trypsin (1–2 mL) and placed in CO_2 incubator the for five minutes. A volume of 3 mL of DMEM was added to stop the reaction of trypsin. The cells were then collected in a centrifuge tube. The centrifuge tube was centrifuged at 1200 rpm for 5 min and the supernatant was discarded. The cells were resuspended in 1 mL of DMEM. 100 μL of cells was plated at a density of 1×10^6 cells/mL into 96-well microtitre plate in triplicate and incubated until