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Biocompatibility of folate-modified chitosan nanoparticles

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ABSTRACT

Objective: To evaluate the acute toxicity of carboxymethyl chitosan-2, 2′ ethylenedioxy bis-ethylamine-folate (CMC-EDBE-FA) and as well as possible effect on microbial growth and *in vitro* cell cyto-toxicity. **Methods:** CMC-EDBE-FA was prepared on basis of carboxymethyl chitosan tagged with folic acid by covalently linkage through 2, 2′ ethylenedioxy bis-ethylamine. *In vivo* acute toxicity, *in vitro* cyto-toxicity and antimicrobial activity of CMC-EDBE-FA nanoparticle were determined. **Results:** Vancomycin exhibited the antibacterial activity against vancomycin sensitive *Staphylococcus aureus*, but CMC-EDBE-FA nanoparticle did not give any antibacterial activity as evidenced by minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC), disc agar diffusion (DAD) and killing kinetic assay. Further, the CMC-EDBE-FA nanoparticle showed no signs of *in vivo* acute toxicity up to a dose level of 1 000 mg/kg p.o., and as well as *in vitro* cyto-toxicity up to 250 μg/mL. **Conclusions:** These findings suggest that CMC-EDBE-FA nanoparticle is expected to be safe for biomedical applications.

1. Introduction

Chitosan (CS) is the deacetylated form of chitin. CS is a linear polysaccharide, composed of glucosamine and N-acetyl glucosamine linked in a β linkage. CS has been reported to possess immune stimulating properties such as promoting resistance to bacterial infection, increasing accumulation and activation of macrophages and polymorphonuclear leukocytes, suppressing tumor growth, augmenting antibody responses and inducing production of cytokines[1]. Utilization of CS derived from crustacean shells may cause hypersensitivity reactions in individual with shellfish allergy[2]. Carboxymethyl chitosan (CMC) is a linear polysaccharide composed of β (1, 4) glycosidic linkages between 6-carboxymethyl-D-glucosamine monomers. CMC is synthesized from CS by carboxylation of the hydroxyl and amine groups. CMC is a water-soluble and biodegradable polymer. Amino and carboxyl functional group of CMC can serve as chelation sites and form complexes with pharmaceuticals[3]. CMC demonstrates potential applications in biotechnology, biomedicine, food ingredients and cosmetics[4,5]. In our previous laboratory report, we synthesized carboxymethyl chitosan-2, 2′ ethylenedioxy bis-ethylamine-folate (CMC-EDBE-FA) nanoparticle based on carboxy methyl chitosan tagged with folic acid by covalently linkage through 2, 2′ (ethylenedioxy) bis-(ethylamine) and established that it has no toxic effect[6,7].

The present study was undertaken to evaluate the acute toxicity of CMC-EDBE-FA and as well as possible effect on microbial growth and *in vitro* cell cyto-toxicity.

2. Materials and methods

2.1 Chemicals and reagents

Folic acid (FA), chitosan (medium molecular weight), dicyclohexyl carbodiimide (DCC), trifluoroacetic acid, 2, 2′-(ethylenedioxy)-bis-(ethylamine) (EDBE), di-tert-butyldicarbonate (BoC_2O), N-hydroxysuccinimide (NHS) and 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride (EDC) were procured from Sigma (St. Louis, MO, USA). Sodium chloride, Luria broth, nutrient broth, nutrient agar, tryptic soy broth, agar powder, Mueller-Hinton broth (MHB), cell culture grade dimethyl sulfoxide (DMSO), RPMI-1640, Dulbecco modified Eagle's medium (DMEM) and minimal essential medium (MEM), fetal calf

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serum, penicillin, streptomycin were purchased from Himedia, India. KH_2PO_4, K_2HPO_4, formaldehyde, alcohol and other chemicals were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. All other chemicals were from Merck Ltd., SRL Pvt., Ltd., Mumbai and were of the highest purity grade available.

2.2. Animals

Acute toxicity experiment was performed using Swiss male mice aged 6-8 weeks old, weighing (20-25 g). The animals were fed standard pellet diet with vitamins, antibiotic and water was given *ad libitum* and housed in polypropylene cage (Tarson) in the departmental animal house with 12 h light: dark cycle under standard temperature (25±2 ℃). The animals were allowed to acclimatize for one week. The animals used in this study did not show any sign of malignancy or other pathological processes. Animals were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India, and approved by the Ethical Committee of Vidyasagar University.

2.3. Bacterial strains

Six vancomycin sensitive *Staphylococcus aureus* (*S. aureus*) strains were clinically isolated from post operative pus samples of patients admitted to Burn and Wound Section of Midnapore Medical College and Hospital, Midnapore, West Bengal, India during a three month period from December 15, 2008 to June 15, 2009[8]. These samples were used in this study to evaluate whether or not CMC-EDBE-FA nanoparticle has antimicrobial activity. Bacterial culture was done in Mueller-Hinton broth at 37 ℃ throughout the experiment.

2.4. Preparation of CMC-EDBE-FA nanoparticle

CMC-EDBE-FA was synthesized *via* reaction of the carboxyl group of carboxymethyl chitosan with the primary amine group of FA-EDBE in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). EDC, a coupling cross linker, first reacts with the carboxyl group of carboxymethyl chitosan to form an active ester intermediate. The formed intermediate can react with the primary amine of FA-EDBE to form an amide bond. The formation of amide bond between carboxymethyl chitosan and FA-EDBE was confirmed from fourier transform infrared spectroscopy (FTIR) and ^{1}H NMR spectrum. Characterization of CMC-EDBE-FA was carried out by ^{1}H NMR, FTIR spectrum, transmission electron microscope (TEM) and dynamic light scattering (DLS) study[6].

2.5. In vivo acute toxicity determination study

Acute toxicity study was performed as per OECD-423 guidelines (acute toxic class method)[9]. Swiss male mice (n=6) selected by random sampling technique were used for acute toxicity study. The animals were kept fasting for overnight providing only water, after which the CMC-EDBE-FA nanoparticle was injected intraperitoneally (i.p.) at the dose level of 5 mg/kg bw and observed for 14 days. If mortality was observed in 2 out of 3 animals, then the dose injected was assigned as toxic dose. If mortality was observed in 1 animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for further higher doses such as 100, 250, 500 and 1 000 mg/kg bw.

2.6. In vitro cyto-toxicity of nanoparticles by 3-(4, 5-dimethylthiazol)-2-diphenyltertrazolium bromide (MTT) assay

The HeLa cell lines were cultivated for *in vitro* experiments. Cell lines were obtained from the National Centre for Cell Sciences (NCCS) Pune, India. It was cultured in DMEM and MEM supplemented with 10% fetal calf serum, 100 units/mL penicillin and 100 μg/mL streptomycin, 4 mM L-glutamine under 5% CO_2 and 95% humidified atmosphere at 37 ℃. HeLa cell lines were seeded into 96 wells of tissue culture plates having 180 μL of complete media and were incubated for 18 h. CMC-EDBE-FA nanoparticle was added to the cells at different concentrations and the mixture was incubated for 72 h at 37 ℃ in a humidified incubator maintained with 5% CO_2. The cell viability was estimated by MTT assay[6].

2.7. Dose of vancomycin and CMC-EDBE-FA nanoparticle for antimicrobial study

Several doses of vancomycin and CMC-EDBE-FA nanoparticle (1.0 μg/mL-100 mg/mL) were prepared using sterile PBS (pH 7.4). In this study, all these doses were charged against vancomycin sensitive *S. aureus*.

2.8. Determination of minimum inhibitory concentration (MIC)

The MIC values of vancomycin and CMC-EDBE-FA nanoparticle were determined by a broth dilution method using MHB[10]. About 5×10^4 cells in MHB were treated with different concentrations of vancomycin and CMC-EDBE-FA nanoparticle and shaken for 16 h at 37 ℃. The minimum concentration at which there was no visible turbidity was taken as the MIC value.

2.9. Determination of minimum bactericidal concentration (MBC)

The MBC values of vancomycin and CMC-EDBE-FA nanoparticle were determined according to our previous laboratory report[10]. This is an extension of the MIC procedure. Vancomycin and CMC-EDBE-FA nanoparticle treated bacterial culture showing growth or no growth in the MIC tests was used for this test. Bacterial culture used for the MIC test was inoculated onto the Mueller-Hinton agar and incubated at 37 ℃ for 24 h. Microbial growth or death was ascertained *via* no growth on Mueller-Hinton agar plate. The minimal concentration of the vancomycin and CMC-EDBE-FA nanoparticle that produced total cell death is the MBC.

2.10. Disc agar diffusion (DAD) test

Susceptibility of isolates to vancomycin and CMC-EDBE-FA nanoparticle was determined by the DAD technique according to Acar *et al* and Bauer *et al*[11,12]. The test bacterium taken from an overnight culture (inoculated from a single colony) was freshly grown for 4 h having approximately 10^6 CFU/mL. With this culture, a bacterial lawn was prepared on Mueller-Hinton agar. Filter paper discs of 6-mm size were used to observe susceptibility patterns against vancomycin and CMC-EDBE-FA nanoparticle [amount of antibiotic per disc in microgram (μg); vancomycin (30) and CMC-EDBE-FA nanoparticle (1, 10, 10^2, 10^3, 10^4, and 10^5)]. Vancomycin disc was obtained commercially from Himedia and CMC-EDBE-FA nanoparticle discs were prepared according to the standard method[12]. The diameter of zone of bacterial growth inhibition surrounding the disc (including the disc), was measured and compared with vancomycin standard. This gave a profile of drug susceptibility vis-à-vis antibiotic resistance[12].

2.11. Killing kinetic studies

Killing kinetic assay of vancomycin sensitive *S. aureus* cell against vancomycin and CMC-EDBE-FA nanoparticle was studied by standard procedure[13]. Vancomycin (1 × MIC value) and CMC-EDBE-FA nanoparticle (1.0 μg/mL and 100 mg/mL) treated with vancomycin sensitive *S. aureus* suspension (10^7 CFU/mL) were grown in sterile glass tubes containing 1 mL of MHB, incubated at 37 ℃ in a shaking water bath. The number of viable organisms was determined as total number of colonies by dilution plating method of broth on MHA after 0, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 h of incubation. The detection limit was 2 $\log_{10}$ CFU/mL.

3. Results

3.1. Characterization of CMC-EDBE-FA

The peak assignment of CMC was as follows, 1 741 cm^{-1} (-COOH), 1 070-1 136 cm^{-1} (-C-O) and 1 624 and 1 506 cm^{-1} ($-NH^{3+}$). FA-EDBE showed the characteristic absorption bands at 1 650 and 1 550 cm^{-1} located in the zone related to the (-CONH-), corresponding to the (C=O) stretching band and to the (-NH) bending vibration band, respectively. The presence of these two bands indicates that an amide bond has been formed between -COOH of folic acid and the-NH_2 amine end group of EDBE. The more characteristics of these two bands have become more prominent and intense in CMC-EDBE-FA. This provides evidence for the formation of an extra amide bond during the attachment of folic acid. ^{1}H NMR spectrum of CMC-EDBE-FA showed the peaks at about 1.9 ppm attributed to the methyl hydrogen of acetamido-2-deoxy-β-D-glucopyranosyl unit; the peaks at about 2.9-3.2 ppm attributed to methylene hydrogen atoms of EDBE and 3.5-4 ppm observed the glucopyranosyl hydrogen atoms. It was clear the proton peaks of 8.7, 7.6, 6.9, 6.4 ppm were observed in ^{1}H NMR spectrum of CMC-EDBE-FA. No such peaks were observed in the same chemical shifts of ^{1}H NMR spectrum for CMC. The appearance of these peaks confirms the successful conjugation of FA-EDBE with CMC. The size of CMC-EDBE-FA self-assembled nanoparticles in aqueous medium measured by DLS ranged from (210± 40 nm). The morphology of CMC-EDBE-FA self-aggregated nanoparticles was investigated by TEM. The nanoaggregate shows a spherical geometry and having a uniform size. At lower magnification nanoparticles having an average size of about 50 nm were observed (figures are not shown)[6].

3.2. Acute toxicity study

CMC-EDBE-FA nanoparticle did not cause any mortality up to 1 000 mg/kg and was considered as safe (X-unclassified)[14].

3.3. In vitro cyto-toxicity assay

The cyto-toxic activity of CMC-EDBE-FA nanoparticle on HeLa cell lines was evaluated by assessing the cell viability using a standard MTT assay method (Figure 1). Different concentrations of CMC-EDBE-FA nanoparticle (5 μg/mL to 25 μg/mL) were added to cells for 24 h. It is found that there was no significant difference in cell viability between the cells treated with CMC-EDBE-FA nanoparticle. Hence this CMC-EDBE-FA nanoparticle is expected to be safe for biomedical applications.

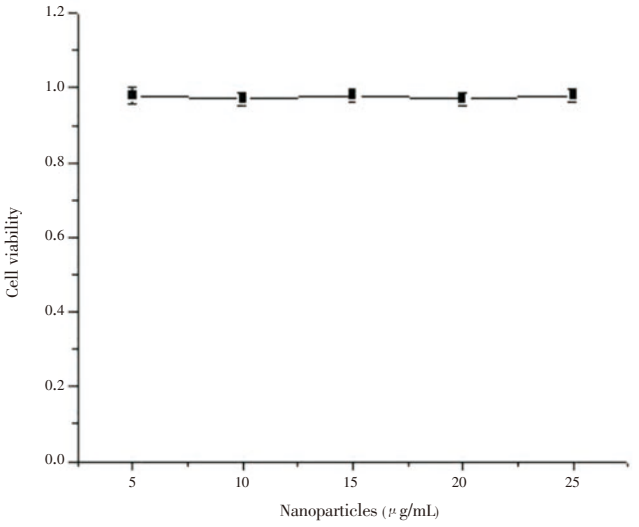

Figure 1. Cytotoxicity of CMC-EDBE-FA nanoparticles (5 to 25 μg/mL) against HeLa cells after 24 h of incubation.

3.4. MIC of vancomycin and CMC-EDBE-FA nanoparticle

The MIC values of vancomycin and CMC-EDBE-FA nanoparticle for vancomycin sensitive *S. aureus* isolates were determined. The MIC value of vancomycin was 10 μg/mL, whereas CMC-EDBE-FA nanoparticle did not give any growth inhibitory effect. In each set of experiment, bacterial control tubes showed no growth inhibition (Figure 2).

3.5. MBC of vancomycin and CMC-EDBE-FA nanoparticle

The MBC values of vancomycin and CMC-EDBE-FA nanoparticle for vancomycin sensitive *S. aureus* isolates were determined. The MBC value of vancomycin was 10 μg/mL, whereas CMC-EDBE-FA nanoparticle did not give any bactericidal effect. In each set of experiment, bacterial control plates showed no growth inhibition (Figure 3).

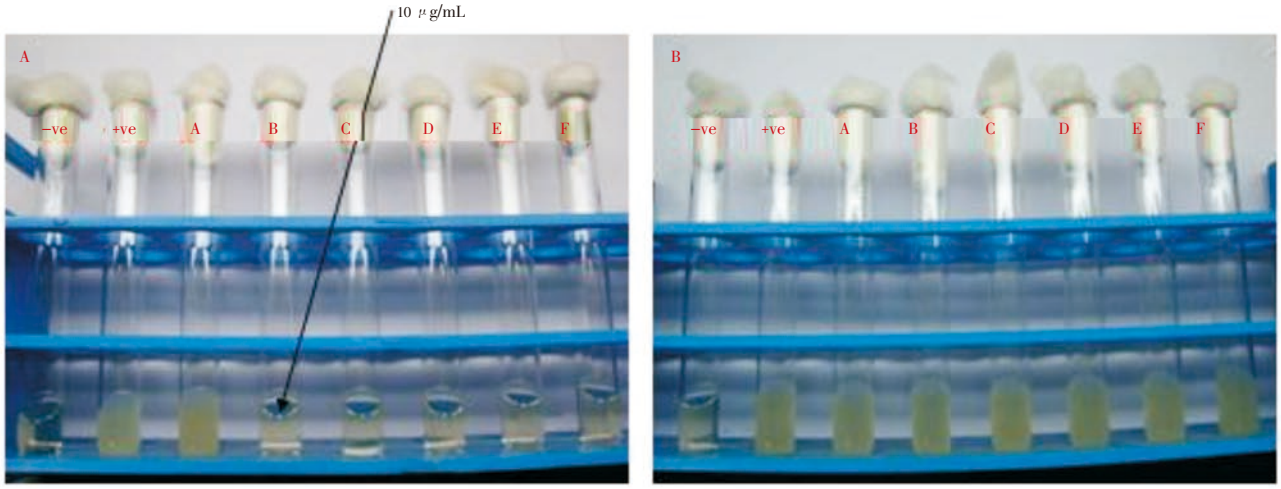

Figure 2. Determination of MIC value of vancomycin (A) and CMC-EDBE-FA nanoparticle (B) against *S. aureus*.
−ve: negative control; +ve: positive control; A: 1.0 μg/mL; B: 10 μg/mL; C: 10^2 μg/mL; D: 10^3 μg/mL; E: 10^4 μg/mL; F: 10^5 μg/mL.

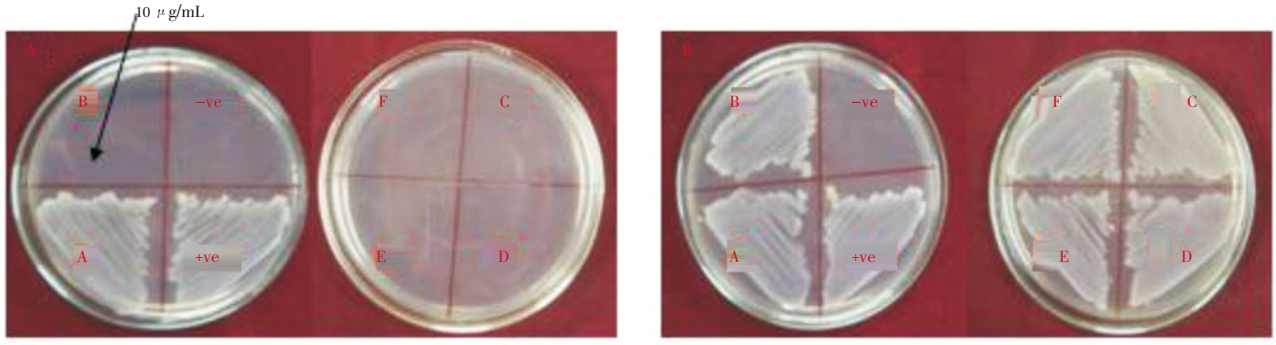

Figure 3. Determination of MBC value of vancomycin (A) and CMC-EDBE-FA nanoparticle (B) against *S. aureus*.
−ve: negative control; +ve: positive control; A: 1.0 μg/mL; B: 10 μg/mL; C: 10^2 μg/mL; D: 10^3 μg/mL; E: 10^4 μg/mL; F: 10^5 μg/mL.

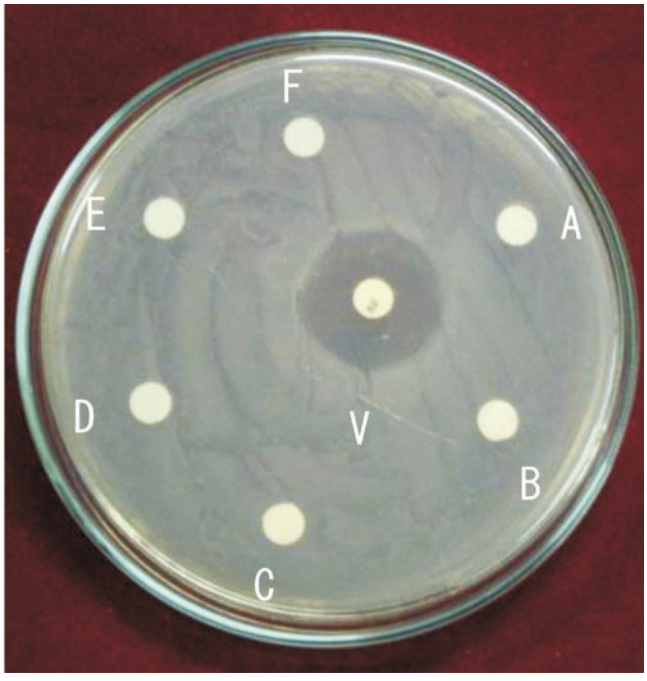

Figure 4. Determination of zone of inhibition of *S. aureus* against vancomycin and CMC-EDBE-FA nanoparticle by disc agar diffusion test.
V: vancomycin; A: 1.0 μg/mL; B: 10 μg/mL; C: 10^2 μg/mL; D: 10^3 μg/mL; E: 10^4 μg/mL; F: 10^5 μg/mL.

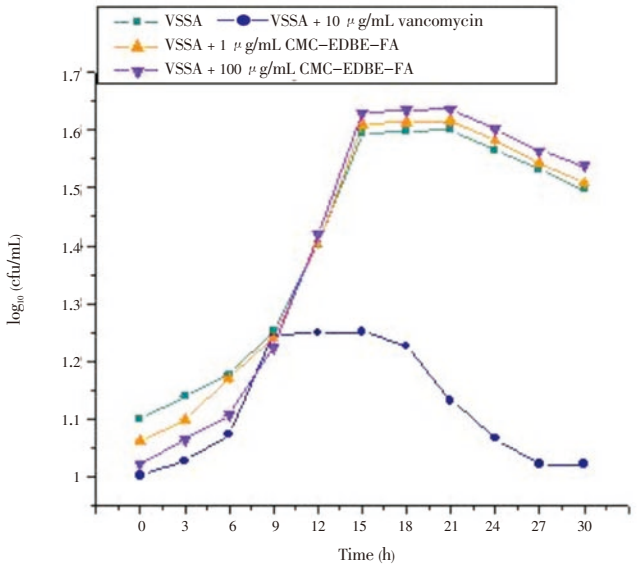

Figure 5. Killing kinetic assay of vancomycin and CMC-EDBE-FA nanoparticle against *S. aureus*.
VSSA: vancomycin sensitive *S. aureus*.

3.6. DAD test

The antibiotic-resistance profile, as determined by DAD test, revealed that vancomycin yielded 20 mm clear zone surrounding the disc, whereas CMC-EDBE-FA nanoparticle discs did not produce any clear zone (Figure 4).

3.7. Killing kinetic studies

The time-kill curves of vancomycin and CMC-EDBE-FA nanoparticle against VSSA were shown in Figure 5. Bactericidal activity (>37.3% reduction) was observed after 12 h exposure of the isolates to vancomycin at MIC concentration, whereas CMC-EDBE-FA nanoparticle did not produce any bactericidal activity, as shown in time-kill curves.

4. Discussion

CMC-EDBE-FA nanoparticle was prepared by the carboxylic group (-COOH) of folic acid and -COOH group of functionalized CMC connected through the end-amino groups hydrophilic spacer, 2,2′-(ethylenedioxy)-bis-ethylamine. It is well known that CMC is easily soluble in water but folic acid is less soluble in water. When CMC is connected by folic acid through a spacer, CMC may act as a hydrophilic part and folic acid as a hydrophobic part.

The results of the present study demonstrated that, vancomycin exhibited bactericidal activity to vancomycin sensitive *S. aureus* cell, followed by bacteriostatic activity. It may be due to the penetration of vancomycin into the bacterial cell which may be followed by the bacteriostatic activity of vancomycin. But CMC-EDBE-FA nanoparticle charged against vancomycin sensitive *S. aureus* strains does not give any antimicrobial activity. It suggests that CMC-EDBE-FA nanoparticle has no antimicrobial effect on vancomycin sensitive *S. aureus*.

It was also found in our study that there was no significant difference in cell viability between the cells treated with CMC-EDBE-FA nanoparticles. Further, the acute toxicity study with the nanoparticle showed no sign of toxicity up to a dose level of 1000 mg/kg p.o., indicating it is safe for applications.

In summary, our present data with both *in vivo* acute toxicity test, *in vitro* cyto-toxicity test and antimicrobial activity study clearly demonstrated that CMC-EDBE-FA nanoparticle has no lethal activity as well as antimicrobial activity. Hence, this CMC-EDBE-FA nanoparticle is expected to be safe for biomedical applications.

Conflict of interest statement

We declare that we have no conflict of interest.

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