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Novel carboxymethyl chitin nanoparticles for cancer drug delivery applications

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ABSTRACT

Carboxymethyl chitin (CMC) nanoparticles were prepared by cross-linking of CMC solution with $CaCl_2$ and $FeCl_3$. The cytotoxicity of the CMC nanoparticles was evaluated using MTT assay and they were found to be non-toxic into L929 mouse cells. The antibacterial and magnetic properties of the CMC nanoparticles were also studied. The prepared CMC nanoparticles were characterized using SEM and FTIR. The model hydrophobic anticancer drug 5-fluorouracil (5-Fu) was loaded into CMC nanoparticles via emulsion cross-linking method. The encapsulation efficiency and *in-vitro* drug release behaviour of drug-loaded nanoparticles were studied by UV spectrophotometer. Drug release studies showed that the CMC nanoparticles showed controlled and sustained drug release at pH 6.8. Moreover, the prepared nanoparticles were also found to be antibacterial and their magnetic properties reveals for their potential use in drug tracking. These results indicated that CMC nanoparticles are a promising carrier system for controlled drug delivery.

1. Introduction

Chitin and chitosan have been shown to be useful natural biopolymers (Inez, Lubben, Verhoef, Borchard, & Junginger, 2001; Kishimoto & Tamaki, 1987; Ouchi, Banba, Fujimoto, & Hamamoto, 1989a; Ouchi, Banba, Matsumoto, Suzuki, & Suzuki, 1989b; Yoshino, Machida, Onishi, & Nagai, 1996) because they possess good biocompatibility and biodegradability (Amano & Ito, 1978; Nakamura, Onishi, Machida, & Nagai, 1992; Pangburn, Trescony, & Heller, 1982). Chitin has been utilized as a material for wound-healing dressings (Kishimoto & Tamaki, 1987). Recently, chitin and chitosan derivatives have been examined as possibly useful drug carriers (Inez et al., 2001; Mi, Chen, Tseng, Kuan, & Shyu, 1997; Ouchi et al., 1989a, 1989b; Song, Onishi, & Nagai, 1992, 1993a, 1993b, 1993c; Song, Onishi, Machida, & Nagai, 1995; Watanabe, Saiki, Uraki, Tokura, & Azuma, 1990; Watanabe et al., 1992), and were generally found to display low toxicity (Inez et al., 2001; Song et al., 1993b, 1995). However, chitosan has been reported to damage cells (Carreno-Gomez & Duncan, 1997; Lee, Ha, & Park, 1995) including lysis of red blood cells (Yasutomi, Nakakita, Shioya, & Kuroyanagi, 1993). On the other hand, CMC is a water-soluble anionic derivative of chitin containing carboxyl groups possess low toxicity (Song et al., 1993b, 1993c). CMC is utilized as a component of wound-healing dressings (Yasutomi et al., 1993), and exhibits high biocompatibility (Song et al., 1993c, 1995). CMC can be synthesized from chitin (Muzzarelli, 1988).

Polymers used, as drug carriers are required to be biodegradable to prevent polymer accumulation in the body (Jayakumar, Nwe, Tokura, & Tamura, 2007; Jayakumar, Prabaharan, Reis, & Mano, 2005; Prabaharan & Mano, 2005). However, since the rate of biodegradation affects drug action as well as toxicity, it must be carefully controlled. CMC generally degrades enzymatically faster than chitosan or *N*-succinyl chitosan (Hata, Onishi, & Machida, 1999; Kamiyama, Onishi, & Machida, 1999; Nakamura et al., 1992; Nishimura, Nishi, & Tokura, 1986). In a previous study, the biodegradability of CMC was examined in detail *in-vitro* and *in-vivo* (Hata et al., 1999). Although the biodegradability of CMC was suppressed by deacetylation, even 30% deacetylated CMC was enzymatically degraded rather fast (Hata et al., 1999). In order to slow down the biodegradation rate of CMC, further increased deacetylation or solidification of CMC are interesting strategies. CMC is known to be precipitated in the coupling with mitomycin C using water-soluble carbodimide, and the particles obtained by grinding of the precipitate exhibited a fairly good retention in the body (Song et al., 1993a, 1993c). However, the particles prepared by the precipitation and subsequent grinding showed an irregular shape and somewhat large particle size (a mean diameter of 7.1 μm) with a wide size distribution (the diameter

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ranging from 1 to 15 μm). These particle characteristics, that is, non-uniform shape and wide size distribution, are unfavourable for drug delivery utilizing tissue localization dependent on the particle size (Kanke, Simmons, Weiss, Bivins, & Deluca, 1980; Sugibayashi et al., 1979; Yoshioka, Hashida, Muranishi, & Sezaki, 1981). Generally, less complex shapes such as spheres are required to minimize the complex interaction of the particles with blood vessels or tissues.

In addition, particles with narrower size distributions are necessary for elective passive targeting; for example, at diameter of 0.2–0.3 μm is needed for effective passive liver targeting, and the particles with a diameter of more than 7 μm are trapped in the lung and remain there over long periods of time (Kanke et al., 1980; Sugibayashi et al., 1979; Yoshioka et al., 1981). Complexation with iron (III) is known as another solidification technique of CMC (Mi et al., 1997; Watanabe et al., 1990, 1992). In these reports, CMC and iron (III) (Hata, Onishi, & Machida, 2000) were directly mixed in aqueous solution to prepare a solid complex and the particle size of the obtained solid was strongly influenced by the preparative conditions. Generally, an emulsification technique is often utilized for the preparation of microcapsules or microspheres.

In this paper, we have taken the approach of cross-linking the CMC as away to control its biodegradability and to result in particles in the nanometer regime. This method potentially provides a most favourable option for drug delivery applications. 5-Fluorouracil (5-Fu) has been in clinical practice for decades as one of the oldest and anteneoplastic chemotherapy drugs. However, current chemotherapy approaches come along with several side effects including diarrhoea, low white blood cell counts, low platelet counts and anaemia etc. Therefore, the life quality of patients under going chemotherapy treatment often deteriorates within a rather short period (Zhang et al., 2008). The main reason is that both target cancerous cells and normal cells are subject to non-selective exposure to the drug, which leads to unwanted toxic side effects. In this work, we report on newly developed CMC nanoparticles for cancer drug delivery applications.

2. Materials and methods

2.1. Materials

Carboxymethyl chitin (degree of substitution – 0.67, degree of deacetylation – 28.7%, viscosity – 15 mPa) was purchased from Koyo Chemicals Co. Ltd., Japan. Calcium chloride ($CaCl_2$), Ferric chloride ($FeCl_3$) and Phosphate Buffer Saline buffer (PBS) were obtained from Sigma–Aldrich USA. 5-Fluorouracil was purchased from Sigma–Aldrich, USA. All other chemicals used were of analytical grades.

2.2. Preparation of CMC nanoparticles

Ten milligrams of CMC was dissolved in 5 ml distilled water. Subsequently, 5 mg of $CaCl_2$ was dissolved in 2.5 ml of distilled water and the mixture was gently added into CMC solution. The resulting solution was stirred continuously for 20 min under mild heating. Then 2.5 mg of $FeCl_3$ was dissolved in 5 ml of distilled water was added into the above solution. This primary solution was stirred for 30 min until the cross-linking process was complete. Next, the nanoparticles were washed with distilled water and purified by repeated centrifugation. The isolated CMC nanoparticles were dried at 40 °C and 760 mm Hg pressure in vacuum oven.

2.3. Preparation of 5-Fu loaded CMC nanoparticles

Drug-loaded nanoparticles were prepared by the emulsion cross-linking method. 5-Fu (3 mg) was dissolved in acetone (5 ml) was added to CMC (50 mg) in distilled water (10 ml) under sonication. This primary emulsion was stirred for 15 min till the organic solvent evaporated. Then the cross-linking agents $CaCl_2$ (15 mg) and $FeCl_3$ (7.5 mg) were dissolved in 5 ml distilled water separately and gently added to the above solution under vigorous stirring. The primary emulsion was stirred continuously for 30 min till the cross-linking was finished. The drug-loaded nanoparticles were isolated using lyophilization technique.

2.4. Characterization techniques

The surface morphology of the CMC nanoparticles was analyzed by scanning electron microscopy (Jeol JSM-649OLA). SEM samples were prepared using platinum sputter coating with a high-resolution coater. Measurements were made using the Sigma Scan Pro software (Jandel Scientific). FTIR spectra of CMC nanoparticles were recorded on a Fourier transform infrared spectrometer (Perkin Elmer, Spectrum RX1). CMC nanoparticles cross-linked with $CaCl_2$ and $FeCl_3$ were prepared as KBr pellets for IR spectroscopy investigations. CMC nanoparticles loaded with 5-flurouracil were analysed accordingly. Magnetic measurements were performed using a Quantum Design DC-SQUID magnetometer (5.5 T-MPMS).

2.5. Cytotoxicity assay

The potential toxicity of CMC nanoparticles against fibroblast cell lines was studied using mouse fibroblast cell line (L929) for cytotoxicity studies. Because, the skin is the first barrier against exposure to a variety of environmental factors. L929 was cultured in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), and 100 U/ml penicillin/streptomycin.

The cytotoxicity of the CMC nanoparticles was evaluated using standard MTT assay by contact method. Mouse Fibroblast cells L929 (obtained from National Centre for Cell Science, Pune, India) were routinely grown in Minimal essential medium with 10% heat-inactivated fetal bovine serum (both from GIBCO-BRL, Gaithersburg, MD) and 1% penicillin/streptomycin (GIBCO-BRL) at 37 °C in a humidified 5% CO_2 atmosphere. A stock solution (3 mg/ml) of the CMC nanoparticles was subsequently diluted using phosphate buffered saline (PBS) (pH 7.2) for treatment. Positive and negative control materials were examined in parallel to validate the test results. The untreated cells were used as a negative control (i.e., 100% viable) and the cells treated with toxin triton were used as positive control. The presence of toxicity was indicated by loss of cell viability. Cells were seeded onto two 96-well plates with a density of 10^4 cells/well and kept in a CO_2 incubator under standard culturing conditions. After 24 h, the culture medium was replaced by 25, 50, 75 and 100% of the stock solution of CMC nanoparticles. The presence of cytotoxic species was verified by a MTT assay after incubating the cells with the CMC nanoparticles for 24 and 48 h. In the assay, the medium was replaced by fresh media containing 10% of MTT and the plate was incubated at 37 °C in a CO_2 incubator for 4 h. Then the medium was removed and, 100 μl of solubilisation buffer (Triton X-100, 0.1 N HCl and isopropanol) was added to each well to dissolve purple formazan crystals. The absorbance of the solution was measured in a microplate reader (Power Wave XS, BioTek) at a wavelength of 570 nm.

2.6. In-vitro antibacterial activity of CMC nanoparticles

The antibacterial activity of CMC nanoparticles was evaluated using the viable cell counting method are described below. Antibacterial studies of the prepared nanoparticles of CMC were performed with ATCC strains of *Staphylococcus aureus* (*S. areus*) (ATCC 25923) using the minimum inhibitory concentration method (MIC). The minimum concentration of the sample that can be

used (MIC) for CMC nanoparticles was 5 ml of a sample with concentration of 3 mg/ml. In this study, the overnight culture of *S. aureus* was taken and turbidity was adjusted to 0.5 McFarland standards. Starting from these parameters, 10 μl of this culture was incubated in a nutrient broth (10 ml) along with three different concentrations (5(A), 10(B) and 15(C) ml of 3 mg/ml) of the above-mentioned nanoparticles and kept in a water bath shaker at 37 °C overnight. These samples were then serially diluted after incubation overnight with 1 mM magnesium sulphate solution up to the 6th dilution and plated on a Nutrient Agar (NA). *S.aureus* without any nanoparticles was plated as a control reference and the plates were kept in an incubator at 37 °C for 12 h. After 12 h of incubation, these plates were observed for colony counting. We determined the antibacterial activity of these nanoparticles based on the number of colonies of the reference vs. samples.

2.7. Drug encapsulation efficiency

The obtained nanoparticles were frozen and lyophilized to obtain a dried nanoparticles product. The weighed product of micelles were washed with PBS solution and then centrifuged, followed by the collection of the supernatant.

The absorbance of the supernatant solution was measured by UV spectroscopy at a wavelength 266 nm and the amount of drug was calculated from a calibration curve that was derived from the absorbance of 5-Fu plotted for different concentrations. The standard equation was calculated as $Y = 0.0329 + 0.0429X$ (Y = absorbance and X = concentration)

$$\text{Encapsulation efficiency} = \frac{\text{Weight of 5-Fu drug in nanoparticles}}{\text{Weight of 5-FU drug initially}} \times 100\%.$$

2.8. In-vitro drug release studies

The *in-vitro* release tests were carried out using 6 wt.% drug-loaded nanoparticles was studied by the following method. Fifty milligrams of each sample were suspended in 40 ml of PBS buffer at pH 6.8 and 37 °C and the samples were placed in an incubated shaker operated at 120 rpm. At predetermined time intervals, 3 ml of aliquots were withdrawn and the concentration of the released drug was monitored by UV spectroscopy (UV-1700 Pharma Spec, Shimadzu) at 266 nm. The dissolution medium was replaced with fresh buffer to maintain the total volume. The drug release percent was determined from the following equation:

$$\text{Drug release}[\%] = C(t)/C(0) \times 100.$$

C(0) and *C(t)* represent the amount of drug loaded and the amount of drug released at the time *t*, respectively. All studies were done in triplicate.

2.9. Evaluation of 5-fu loaded CMC nanoparticles as tumour inhibitor

Cell lines of oral epithelial carcinoma KB cells were routinely grown in minimal essential medium with 10% heat-inactivated fetal bovine serum (both purchased from GIBCO-BRL, Gaithersburg, MD) and 1% penicillin/streptomycin (GIBCO-BRL) at 37 °C in a humidified 5% CO_2 atmosphere. Drug-loaded nanoparticles were used to induce cytotoxicity in the dose indicated. A stock solution (3 mg/ml) of the sample was prepared and subsequently diluted with PBS (pH 7.2) for treatment. Treatments were carried out in 96-well tissue culture plates (BD Bio Science).

Determination of cell viability after treatment with chemicals is the routine method in toxicological assays. The MTT salt (3–4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is reduced by mitochondrial dehydrogenases to the water insoluble MTT formazan. The extracted MTT derivative was mixed with 1 mL of isopropyl alcohol containing 0.04 wt.% HCl, and the absorbance values were determined at a test wavelength 570-nm and a reference wavelength of 630-nm to test the cell viability. After treatment with various concentrations of the material the culture medium was replaced with serum-free medium containing 0.5 mg/mL MTT, and cultures were incubated for an additional 3 h. The blue MTT formazan was dissolved in isopropyl alcohol with the specified percentage of HCl, and their absorbance values were measured using a spectrophotometer. The toxin treated cells were used as positive control and the untreated cells were used as a negative control (i.e., 100% viable), and all values from the experiment are correlated with this data set. We performed two sets of MTT to determine the levels of toxicity.

3. Results and discussion

3.1. Characterization

Fig. 1 shows the surface morphology of the prepared nanoparticles with and without drug. The CMC nanoparticles showed a spherical morphology with diameters in the range of 200–250 nm (Fig. 1a) according to SEM images. The SEM images of the drug-loaded nanoparticles also showed similar morphology (Fig. 1b).

Fig. 2 shows the FTIR spectrum of the prepared nanoparticles. The CMC nanoparticles were cross-linked by calcium and iron ions. The CMC nanoparticles exhibit sharpened peaks due to the interaction of hydroxyl groups (3420 cm^{-1}), carboxylic groups (1065 cm^{-1}) and amide groups (1640 cm^{-1}) with the Ca^{2+} and Fe^{3+} ions, respectively.

In order to compare the spectral changes in the IR spectra between original drug and its complexes, the absorption frequencies of 5-Fu were compared to those of drug-loaded CMC nanoparticles. The strong characteristic absorption of C=O of 5-Fu between 1600

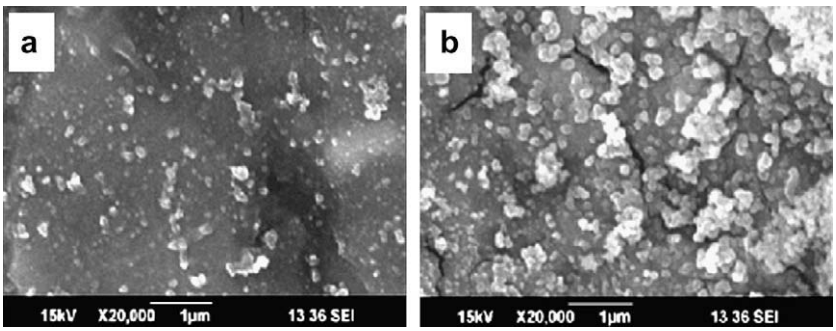

Fig. 1. SEM images of (a) CMC nanoparticles and (b) 5-Fu drug-loaded CMC nanoparticles.

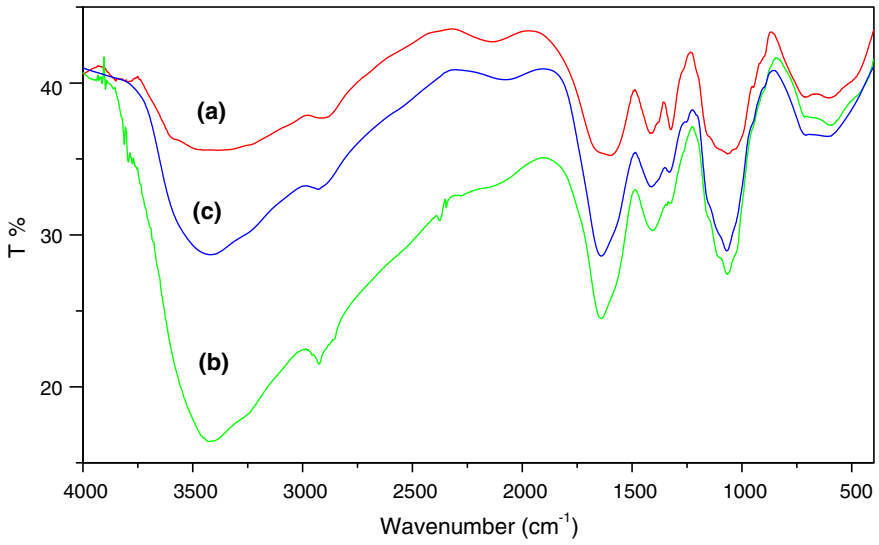

Fig. 2. FTIR spectra of (a) CMC (b) CMC nanoparticles (c) CMC nanoparticles with 5-Fu drug.

and 1720 cm^{-1} was found to be sharper in the FTIR spectrum of the complex (Wang et al., 2003).

The magnetic behaviour of the CMC nanoparticles was examined (Fig. 3) (Yao, Liu, & Risen, 2006) the magnetization (M) as a function of the applied field (H) at 300 K for the as-synthesized CMC nanoparticles. The nanoparticles exhibit quite a weak hysteresis that might be caused by trace amounts of ferromagnetic species (approx. in the range of 1 μg of elemental iron). The major magnetic component of the sample, however, is paramagnetic so that the hysteresis curve is strongly biased. As little is known about

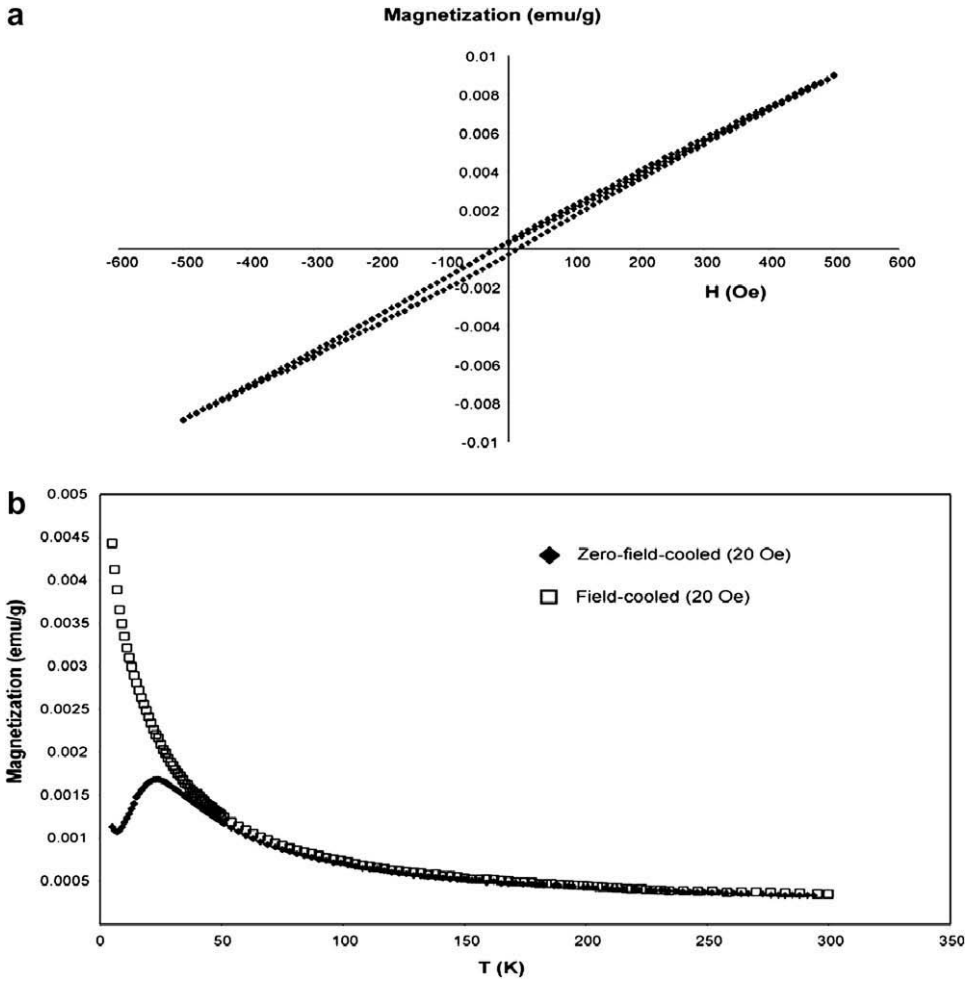

Fig. 3. (a) M–H relationship for CMC nanoparticles at 300 K, (b) temperature dependent magnetization curves (field-cooled and zero-field cooled) at a magnetic field of 20 Oe.

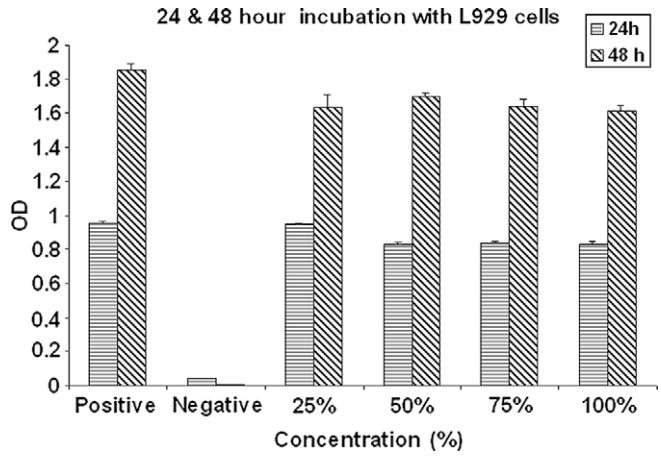

Fig. 4. Cytotoxicity studies of CMC nanoparticles using MTT assay.

the magnetic behaviour of nanoscale iron-crosslinked chitin derivatives, we have performed more detailed measurements of the temperature dependent magnetization (Fig. 3b). The zero-field cooled *M–T* curve recorded at 20 Oe indicates a magnetic hysteresis, stemming probably from a magnetic ordering at low temperatures. Further investigations are in progress in order to understand the complex magnetic interactions within the nanoparticles and to optimize their ferromagnetic properties for drug tracking systems.

3.2. Cytotoxicity assay of CMC nanoparticles

The toxicity of the synthesised CMC nanoparticles was evaluated as described in the aforementioned methods. The optical density (OD) obtained after the MTT assay is directly proportional to number of viable cells. The OD was enhanced after 48 h, as the number of cells increased. No toxicity was observed for any of the concentrations of the extract, including all the possible leachable, and degradation products. Cells were proliferated normally at 48 h compared to 24 h as evident by the MTT assay (Fig. 4). This result suggests that the synthesised CMC nanoparticles do not form any toxic leachable or degradation products and thus used for drug encapsulation applications.

3.3. Antibacterial activity of CMC nanoparticles

The antibacterial activities of the CMC nanoparticles were determined by MIC method with *S. aureus* strain. Fig. 5 is the optical photograph of the plate showing the antibacterial effect of CMC nanoparticles.

Based on the number of colonies of sample and control we calculated the antibacterial activity for the three concentrations of each of the CMC nanoparticle solutions (Fig 6a). It is clearly evident from Fig 6b that, when the concentration of CMC nanoparticles increases (A < B < C), the number of colonies of bacteria decreased significantly. We found that column with maximum CMC nanoparticle concentration "C" shows an absence of bacterial growth. The possible reason for the antimicrobial activity may be the aggressive binding of the nanoparticles onto the microbial cell surface, leading to a gradual shrinkage of the cell membrane and finally to the death of the cell (Prashanth & Tharanathan, 2007). Several other possible explanations have been proposed for the antimicrobial activity of CMC molecules, e.g. interaction with the predominant components of the cell wall (lipopolysaccharides and proteins) of the microorganism. This results in the leakage of intracellular components and also, due to changes in the permeability barrier pre-

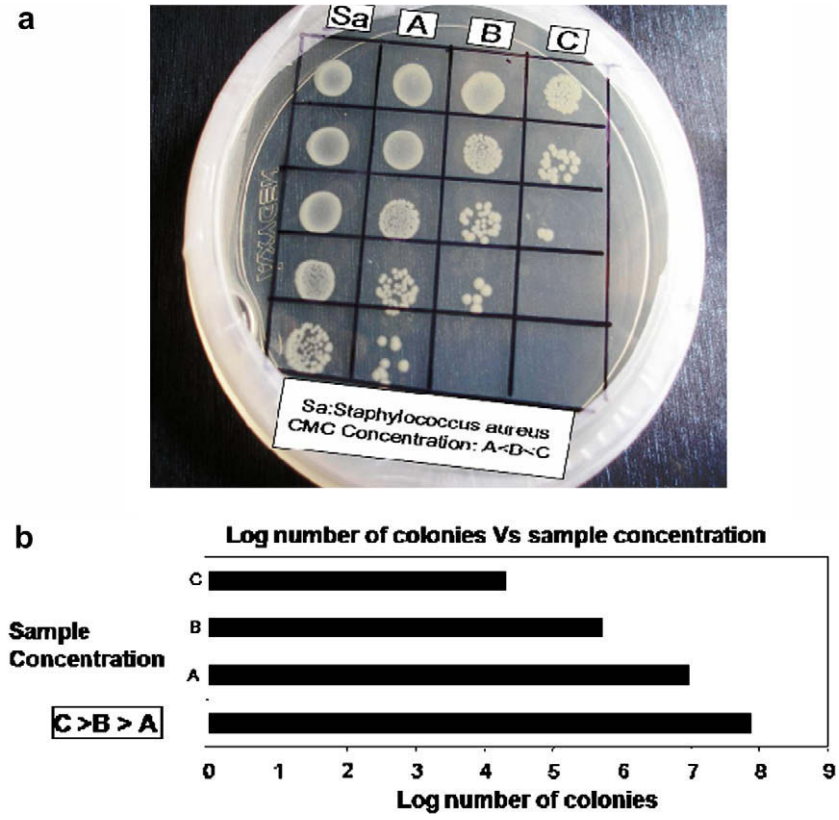

Fig. 5. (a) Representative plate showing the antibacterial activity of CMC nanoparticles (A = 5 ml, B = 10 ml, C = 15 ml of CMC nanoparticles of 3 mg/ml concentration) and (b) Antibacterial activity of CMC nanoparticles (A = 5 ml, B = 10 ml, C = 15 ml of CMC nanoparticles of 3 mg/ml concentration).

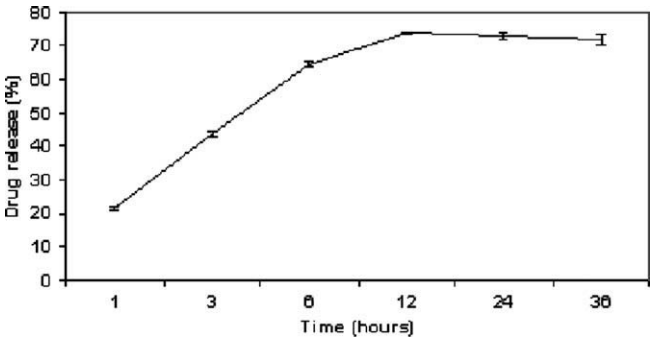

Fig. 6. Release rate of 5-FU drug from CMC nanoparticles.

vents nutrients from entering the cell. Low molecular weight components of the CMC can then enter the cell, bind to DNA through hydrophobic interaction and inhibit protein synthesis (Prashanth & Tharanathan, 2007).

3.4. Drug encapsulation efficiency and in-vitro drug release studies

Drug encapsulation efficiency was determined using UV spectroscopy. The drug encapsulation efficiency was found to be 78.3%. The amount of drug loaded was 6 wt.% of the total base polymer. The release of the drug from polymer micelles is affected by many factors such as polymer degradation, molecular weight, crystallinity, the binding affinity between the polymer and drug, etc. (Shin, Kim, Lee, Cho, & Sung, 1998). The *in-vitro* drug release studies were carried out at pH 6.8. Because, the surrounding of tumour mass has a slightly acidic pH around 6.8. Since our system is carrying an anticancer drug 5-Fu, it was interesting to study how the drug release profiles progress at that particular pH. So we did the drug release experiments at pH 6.8.

Fig. 6 shows the CMC nanoparticles released 5-Fu in a controlled and sustained manner. After 12 h, CMC nanoparticles released 74% of the drug at pH 6.8 and the release percentage was slowly decreasing afterwards.

In this the drug was homogeneously distributed in the CMC nanoparticles. Release occurs by a combination of diffusion of the drug out of the particles into the external environment and also by the degradation of the polymer. The decrease of the drug release at longer time suggested the importance of the diffusion process in the release kinetics. However, the high overall total release is attributed to the ability of the polymer to degrade. The CMC degrades as a result of hydrolysis of the polymer chains into biologically compatible, and progressively smaller compounds. Degradation may take place through bulk hydrolysis, in which the polymer degrades in a fairly uniform manner throughout the matrix.

3.5. Evaluation of 5-Fu loaded CMC nanoparticles as tumour inhibitor

Cell viability assays (Fig. 7) show a reduction in viability with increasing concentrations of 5-Fu loaded CMC in KB cells, represented by solid black bars. However, no significant effect was observed in the drug free CMC cells with the same treatment represented by the soft off-white bars. The toxicity can be seen to increase with increasing loading levels of CMC nanoparticles containing drug. This shows higher drug amounts were released from the CMC nanoparticles with initial drug concentrations over time, resulting in a cytotoxic effect on the cancer cells. This led to a reduction of cell viability and further decrease in OD. These results confirmed that CMC nanoparticles are a very promising candidate for potential cancer drug delivery applications.

4. Conclusions

Carboxymethyl chitin (CMC) nanoparticles were prepared through a cross-linking approach with $FeCl_3$ and $CaCl_2$ and characterized using SEM and FT-IR investigations. The size of the prepared CMC nanoparticles was found to be 200–250 nm, thereby rendering the morphology suitable for drug delivery applications. Furthermore, the CMC nanoparticles display an interesting magnetic behaviour at low temperatures with high optimization potential for their targeted medicinal use. The cytotoxicity studies showed that the prepared CMC nanoparticles are non-toxic against mouse L929 cells. The CMC nanoparticles exhibit significant anti-

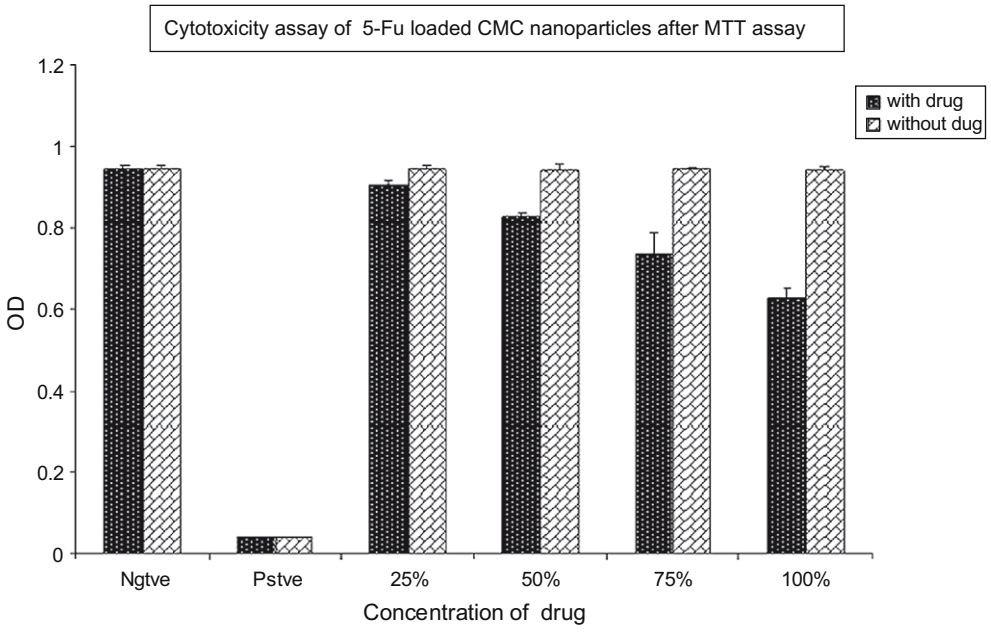

Fig. 7. Cytotoxicity assay of 5-Fu loaded CMC nanoparticle against KB cells.

bacterial activity against *Staphylococcus* bacteria strains. The *in-vitro* studies of the drug-loaded CMC nanoparticles demonstrated a sustained and controlled release of 5-Fu drug at pH 6.8. Therefore, the obtained CMC nanoparticles can be used for controlled drug delivery applications. In addition, 5-Fu drug-loaded CMC nanoparticles display enhanced toxicity against KB cells. The toxicity was found to increase with the initial loading levels. These results suggest that the CMC nanoparticles are a highly promising cancer drug carrier system with interesting magnetic properties that are now subject of further studies.

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