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Short communication

# *O*-Carboxymethyl chitosan nanoparticles for metformin delivery to pancreatic cancer cells

K.S. Snima[a], R. Jayakumar[a], A.G. Unnikrishnan[b], Shantikumar. V. Nair[a], Vinoth-Kumar Lakshmanan[a,*]

[a] *Amrita Centre for Nanosciences and Molecular Medicine, Amrita Institute of Medical Sciences & Research Centre, Amrita Vishwa Vidyapeetham University, Kochi 682041, India*
[b] *Department of Endocrinology, Amrita Institute of Medical Sciences and Research Centre, Amrita Vishwa Vidyapeetham University, Kochi 682041, India*

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ABSTRACT

In this work we developed metformin loaded *O*-carboxymethyl chitosan (*O*-CMC) nanoparticles (NPs) by ionic-gelation method. The prepared NPs of 240 ± 50 nm size with spherical morphology exhibited a pH sensitive release of metformin *in vitro*. Cytotoxicity studies showed that the drug-incorporated NPs induced significant toxicity on pancreatic cancer cells (MiaPaCa-2) compared to normal cells (L929). Metformin loaded NPs exhibited nonspecific internalization by normal and pancreatic cancer cells; however metformin released from the NPs induced preferential toxicity on pancreatic cancer cells. Our preliminary studies suggested that such a novel approach could possibly overcome the current limitations of metformin in its clinical application against pancreatic cancer.

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## 1. Introduction

Pancreatic cancer has the worst mortality rate and lowest overall survival among all cancers with a 5-year survival rate of only 3–6% in US (Siegel, Ward, Brawley, & Jemal, 2011). This type of cancer is difficult to detect, hard to diagnose, early to metastasize; hence it requires an immediate, effective and radical new therapy for treatment in limited time. Prevention of pancreatic cancer is also very important. Metformin, an FDA approved, well-accepted antidiabetic drug has recently been identified as having antagonistic effect on pancreatic cancer proliferation (Kisfalvi, Eibl, Sinnett-Smith, & Rozengurt, 2009; Li, Yeung, Hassan, Konopleva, & Abbruzzese, 2009; Schneider et al., 2001) by inhibition of mTOR (mammalian target of rapamycin), through the activation of AMP kinase (AMPK) (Kisfalvi et al., 2009). Moreover decreased growth of pancreatic cancer xenografts in nude mice models administered with metformin emphasizes its potential to become a pancreatic cancer drug (Kisfalvi et al., 2009). Currently, the low bioavailability and short half-life of metformin (Graham et al., 2011) hinders its application as a full-fledged anti-cancer drug. *O*-CMC, a derivative of chitosan, received considerable attention in drug delivery applications (Anitha et al., 2009; Chen & Park, 2003; Jayakumar et al., 2010). Its reactive *O*-carboxymethyl substitution can interact with $NH_3^+$ group of metformin, facilitating its incorporation during nanoparticle formation. The present study aims in evaluating the ability of *O*-CMC as a carrier for metformin so that it could be a potential therapy or a preventive strategy for pancreatic cancer as well as type 2 diabetes.

## 2. Experimental

### 2.1. Materials

*O*-CMC [molecular weight – 122,000 Da; degree of deacetylation (DDA) – 61.8%; and degree of substitution (DS) – 0.54] was purchased from Koyo chemical Co. Ltd., Japan. Metformin, calcium chloride ($CaCl_2$), rhodamine-123 dye, DAPI stain, minimum essential medium (MEM) and MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium] were purchased from Sigma–Aldrich. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were supplied by Invitrogen. Human pancreatic cancer cell line (MiaPaCa-2) and mouse fibroblast cell line (L929) were purchased from National Center for Cell Sciences (NCCS), Pune, India.

### 2.2. Synthesis of bare and metformin incorporated O-CMC NPs

*O*-CMC NPs were prepared by ionic gelation method as described in the literature (Anitha et al., 2009; Shi, Du, Yang, Zhang, & Sun, 2006). Briefly, 2% (w/v) $CaCl_2$ was added drop wise to 5 mL of 0.5% (w/v) *O*-CMC solution preincubated with 18.75 mg metformin HCl.

* Corresponding author. Tel.: +91 484 2851234; fax: +91 484 2802030.
*E-mail addresses:* vinothlakshmanan@aims.amrita.edu, vinoth.lakshmanan@gmail.com (V.-K. Lakshmanan).

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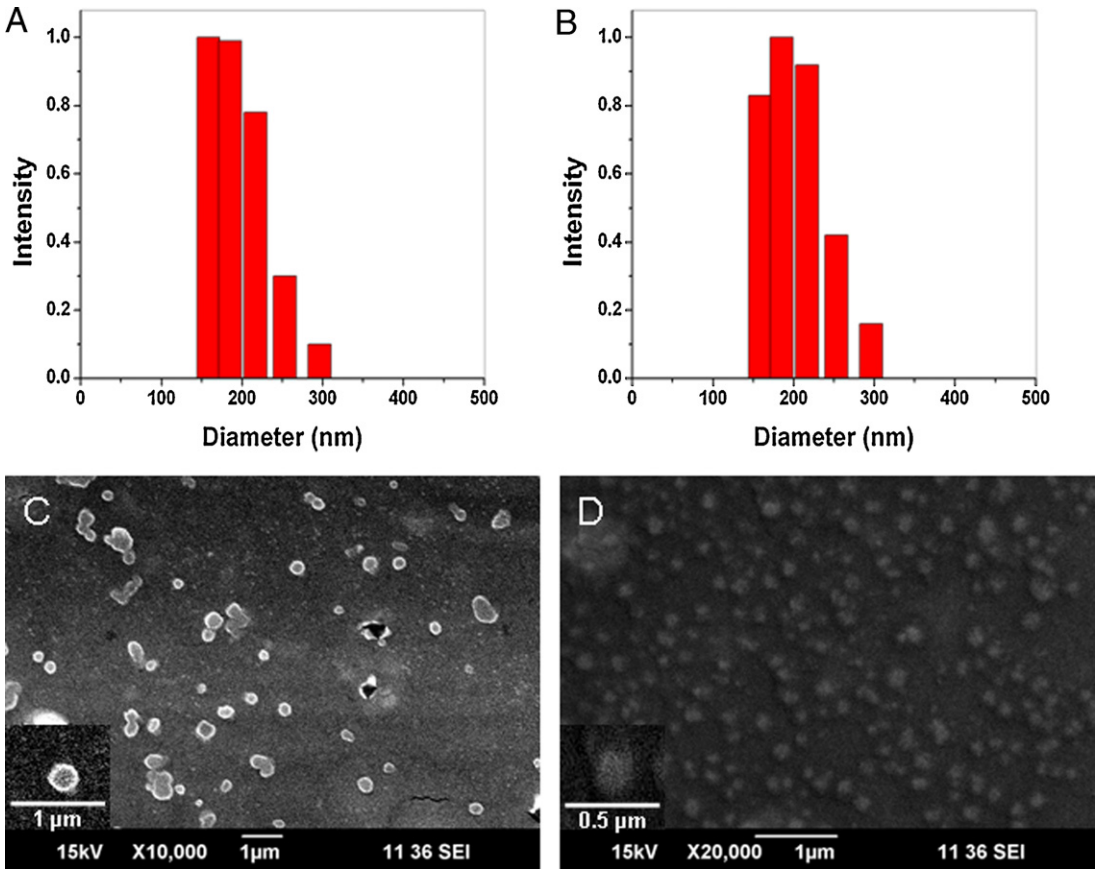


**Fig. 1.** Particle size distribution of bare *O*-CMC NPs (left) and *O*-CMC-metformin NPs (right) by DLS (A and B) and SEM (C and D).

The $CaCl_2$ was added till the solution became turbid. Bare *O*-CMC NPs were prepared similarly without including drug. For *in vitro* studies the nanoparticles were synthesized in laminar air flow hood after sterilizing the components using syringe filter with pore size 0.22 μm.

### 2.3. Labeling of O-CMC-metformin NPs with rhodamine-123

Rhodamine-123 tagged *O*-CMC-metformin NPs were synthesized by incorporating rhodamine-123 into NPs during particle synthesis. 5 mL of 0.5% (w/v) *O*-CMC solution was incubated for 1 h with 18.75 mg metformin HCl and 25 μL of sterile rhodamine-123 (1 mg/mL). Later NPs were prepared by drop wise addition of 2% (w/v) $CaCl_2$ into it till the solution became turbid.

### 2.4. Nanoparticle characterizations

The hydrodynamic diameter of NPs was measured by dynamic light scattering (DLS-ZP/Particle Sizer NicompTM 380 ZLS). The size confirmation and surface morphology analysis of NPs were done using Scanning Electron Microscope (JEOL JSM-6490LA Analytical Scanning Electron Microscope). Surface charge of NPs was recorded from zeta potential measurement using DLS-ZP/Particle Sizer NicompTM 380 ZLS. FT-IR spectra of materials were examined using KBr pellet (1% (w/w) of product in KBr) with a resolution of 4 $cm^{-1}$ and 100 scans per sample on Perklin Elmer Spectrum RXI Fourier Transform Infrared spectrophotometer.

### 2.5. In vitro drug release

The *O*-CMC-metformin NPs suspension containing 3.7 mM metformin was taken in dialysis tubing (molecular weight cut-off = 10 kDa from Sigma–Aldrich) and allowed for dialysis by immersing it into 20 mL phosphate buffered saline (PBS) at pH 7.4 and 4.5. The samples were incubated at 37 °C under shaking (40 rpm). At definite time intervals, 500 μL dissolution medium was taken for the analysis and replaced with fresh medium. The released drug was quantified at 232 nm using spectrophotometer (Shimadzu UV-1700).

Release was calculated as follows:

$$\text{Release}\,(\%) = \frac{\text{Released metformin}}{\text{Total metformin}} \times 100$$

### 2.6. Cytotoxicity studies

MTT assay was performed on L929 and MiaPaCa-2 cells maintained in MEM and DMEM respectively to evaluate cytotoxicity of the prepared NPs. The cells (10,000 cells/$cm^2$) were incubated for 48 h with bare metformin (2, 3 and 3.7 mM), *O*-CMC-metformin NPs (containing 2, 3 and 3.7 mM drug) and bare *O*-CMC NPs (equivalent concentrations). Untreated cells acted as positive control and cells treated with 1% (v/v) Triton X-100 as negative control. The cells were incubated for 4 h with 0.5 mg/mL of MTT followed by 1 h solubilization buffer (10% (v/v) Triton X-100 and 0.1 N HCl in isopropanol) treatment. Then optical density was measured at 570 nm using Beckmann Coulter ELISA plate reader (BioTek Power Wave XS). Cell viability was calculated as:

$$\text{Viability}\,(\%) = \frac{Nt}{Nc} \times 100,$$

where *Nt* is the absorbance of treated cells and *Nc* is the absorbance of untreated cells.

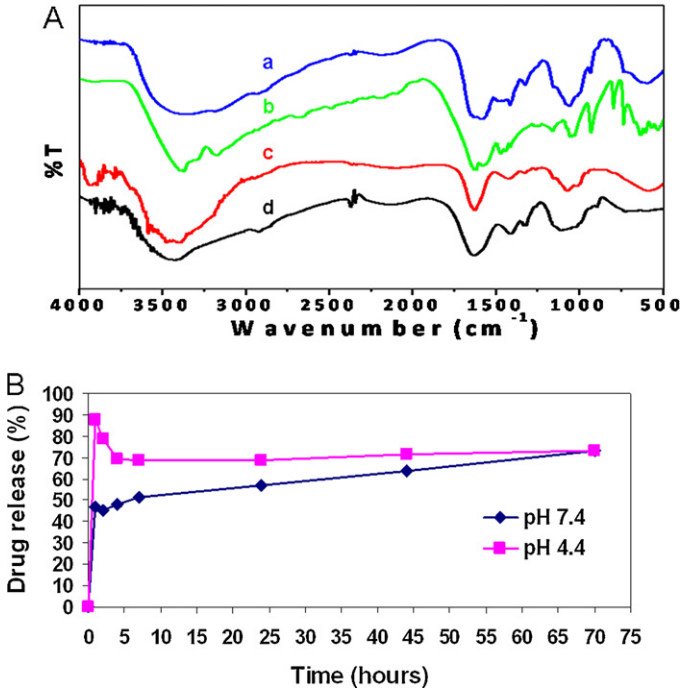


**Fig. 2.** (A) FT-IR spectrum showing *O*-CMC-metformin NPs (a), metformin (b), bare *O*-CMC NPs (c) and *O*-CMC (d). (B) Drug release profile of metformin from *O*-CMC-metformin NPs in pH 4.4 and 7.4 at 37 °C.

### 2.7. In vitro hemolysis assay

Fresh blood was collected from human volunteers into acid citrate dextrose (ACD) containing tubes. For the assay different concentrations of samples were prepared in 0.9% (w/v) saline. 1% (v/v) Triton-X 100 and 0.9% (w/v) saline were kept as positive and negative control respectively. 0.1 mL of the samples was treated with 1 mL of blood and incubated at 37 °C for 5 h at 40 rpm. After incubation, plasma was collected by centrifugation at 4500 rpm for 10 min and optical density at 415, 380 and 450 nm (Anusha et al., 2011) was measured spectrophotometrically using Beckman Coulter Elisa plate reader. Hemolysis was calculated as:

$$\text{Hemolysis}\,(\%) = \frac{\text{Plasma Hb content in test}}{\text{Total Hb content}} \times 100$$

### 2.8. Cell uptake studies

L929 and MiaPaCa-2 cells (15,000 cells/cm$^2$) were grown on cover slips and incubated with rhodamine tagged *O*-CMC-metformin NPs at a concentration containing 3.7 mM metformin for 24 h. After incubation, the cells were fixed using 5% (v/v) paraformaldehyde and were treated with DAPI (300 nM in PBS) for 5 min. Thereafter, coverslips with cells were dried and mounted on to slides using DPX mountant. The samples were analyzed using fluorescent microscope (OLYMPUS DP 71) by keeping untreated DAPI stained cells as control.

## 3. Results and discussion

### 3.1. Preparation and characterization of O-CMC-metformin NPs

*O*-CMC-metformin NPs were successfully prepared using ionic gelation method. The preincubation of metformin with *O*-CMC was carried out to facilitate electrostatic interaction between the amphoteric polymer and cationic metformin. Later the drug was entrapped with in the nanoparticles by crosslinking *O*-CMC using

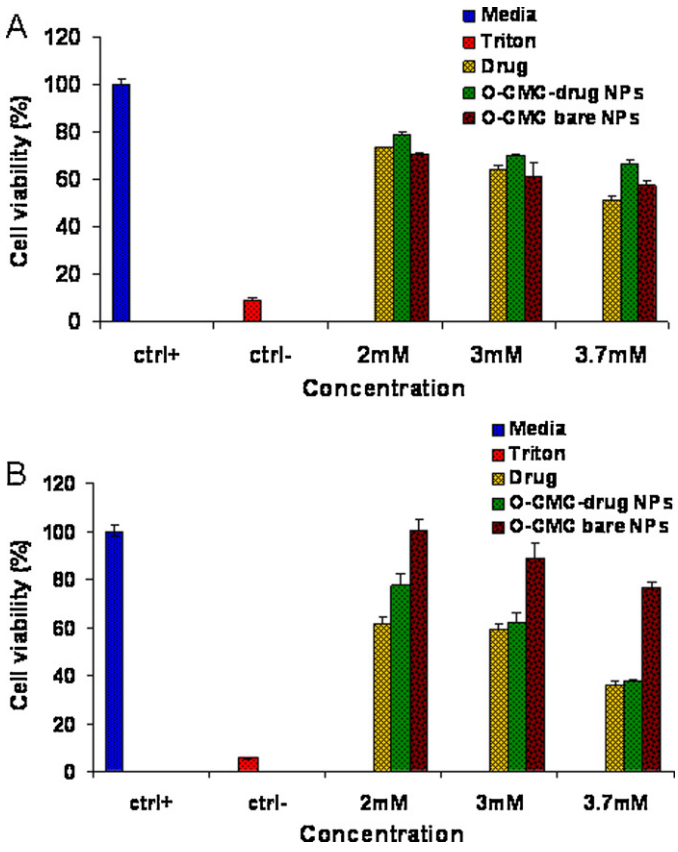


**Fig. 3.** Cell viability of (A) normal cells (L929) and (B) pancreatic cancer cells (MiaPaCa-2) treated with metformin, bare *O*-CMC NPs and *O*-CMC-metformin NPs (48 h incubation).

$CaCl_2$. The hydrophilicity of drug pose difficulty in achieving high entrapment as it can easily come to the aqueous phase outside. So for further studies, the prepared nanoparticle suspension as such has been used.

DLS (Fig. 1(A and B)) and SEM (Fig. 1(C and D)) data showed that the bare *O*-CMC and *O*-CMC-metformin NPs comes in nanometer regime by having size in 230 ± 50 nm. The SEM analysis also revealed spherical morphology of the NPs. The zeta potential analysis of *O*-CMC-metformin NPs revealed a net surface charge of −18.32 mV at pH 7.4. The net negative charge could be attributed by the unreacted carboxyl group of *O*-CMC.

From FTIR spectra (Fig. 2(A)), the peaks at 1626 and 1583 cm$^{-1}$ corresponding to the stretching vibration of C=N and peaks at 936, 800 and 736 cm$^{-1}$ typical for N—H wagging in metformin (Gunasekaran, Natarajan, Ranganayaki, & Natarajan, 2006) indicated the presence of drug within the NPs. Furthermore, the decreased intensity of metformin peaks at 3372 and 3176 cm$^{-1}$ suggested the possibility of hydrogen bonding between the metformin and *O*-CMC in the NPs (Patil, Kuchekar, Chabukswar, & Jagdale, 2010).

### 3.2. In vitro drug release

Fig. 2(B) depicts the *in vitro* drug release profile of metformin from *O*-CMC-metformin NPs at pH 7.4 and 4.4. At neutral pH initial burst release of surface adsorbed drug was observed followed by slow and sustained release of entrapped drug from the NPs. Nearly 50% of drug was released from the NPs within the first 10 h at pH 7.4 and 72% was found released after 70 h. But at acidic pH (pH 4.4) almost 90% of drug was released within the first hour itself indicating a pH sensitive release of metformin from the NPs. The

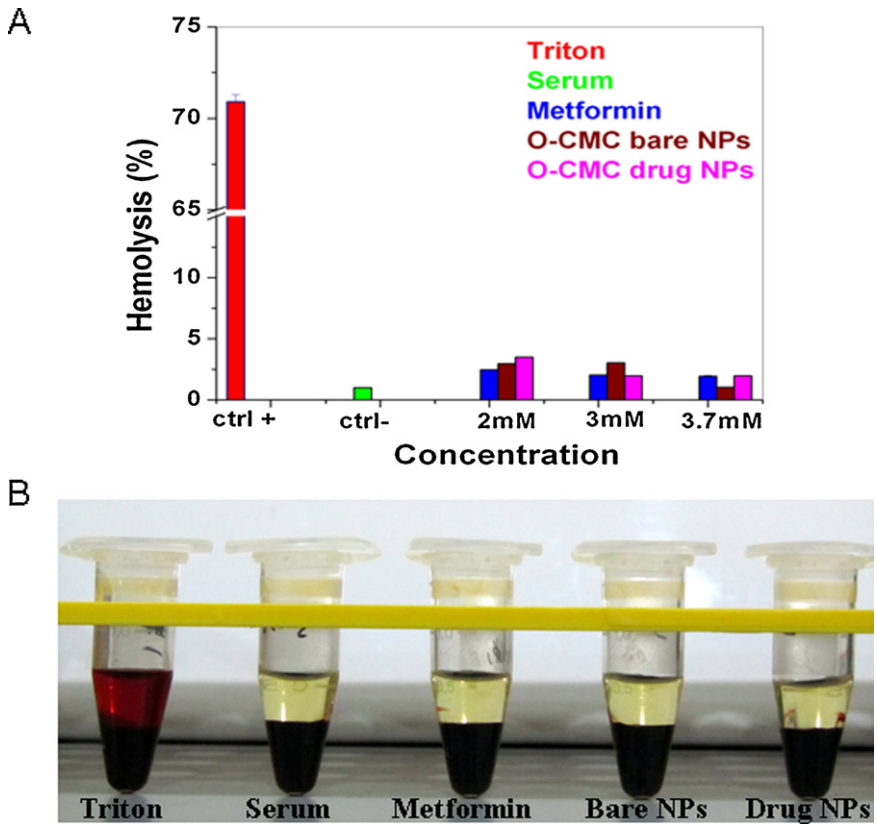


**Fig. 4.** (A) Graph showing % hemolysis *versus* concentration of samples. (B) Hemocompatibility of 3.7 mM metformin, bare *O*-CMC NPs and *O*-CMC-metformin NPs in saline.

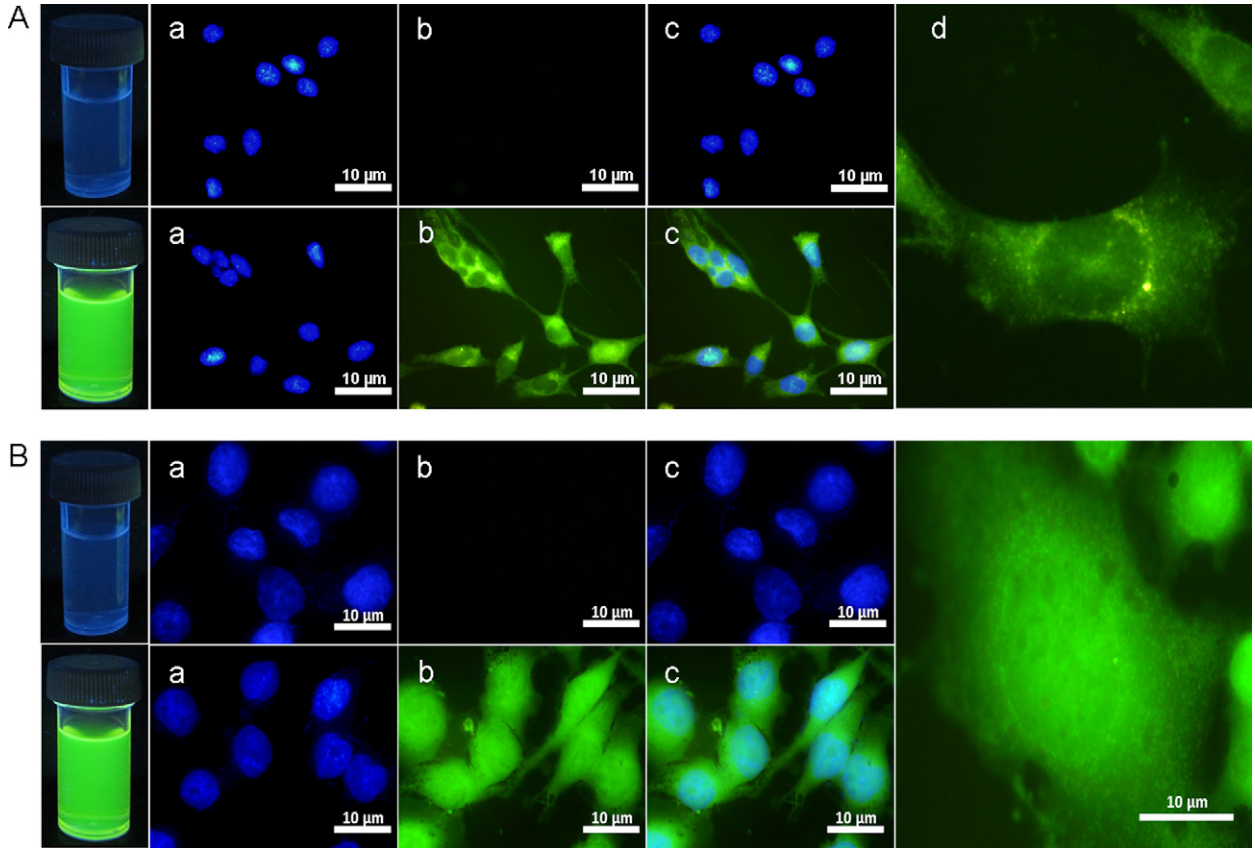


**Fig. 5.** Cellular uptake images of L929 (A) and MiaPaCa-2 (B) cells without treatment (upper panel) and treatment with 3.7 mM rhodamine-123 tagged *O*-CMC-metformin NPs (lower panel), showing DAPI (a), rhodamine (b) and merged (c) images in both panel at 60× magnification. *O*-CMC-metformin NPs treated cells at 100× magnification (d).

enhanced drug release in the acidic pH can be possibly due to the protonation of $NH_2$ groups which resulted in swelling of *O*-CMC NPs (Anitha et al., 2011).

### 3.3. Cytotoxicity studies

Cytotoxicity of nanoformulations on normal cells (L929) and pancreatic cancer cells (MiaPaCa-2) were compared (Fig. 3(A and B)) using MTT assay. The cell viability plot of L929 exposed to metformin and *O*-CMC-metformin NPs showed similar pattern as that exposed to same concentrations of bare *O*-CMC NPs. This indicated that the normal cells were not affected by the toxicity of metformin or *O*-CMC-metformin NPs. At the same time on MiaPaCa-2 cells, metformin and *O*-CMC-metformin NPs induced more toxicity than bare *O*-CMC NPs. Moreover, the highest concentration (3.7 mM) of metformin and *O*-CMC-metformin NPs used in the study showed 40% more toxicity than bare *O*-CMC NPs on MiaPaCa-2 cells. This preferential cytotoxicity could be attributed by the metformin-mediated inhibition of mTOR in pancreatic cancer cells (Kisfalvi et al., 2009). The results also indicated that the anticancer effect of metformin was retained even after incorporating it in to *O*-CMC NPs.

### 3.4. Hemolysis assay

The percentage of hemolysis induced by metformin, bare *O*-CMC NPs and *O*-CMC-metformin NPs (Fig. 4(A)) were less than 5% (critical safe hemolytic ratio for bio-materials according to ISO/TR 7406). Hemolytic property of the highest concentration of samples was shown in Fig. 4(B). The results indicated that the *O*-CMC-metformin NPs are hemocompatible and therefore is safe for intravenous administration.

### 3.5. Cell uptake studies

Internalization of rhodamine-123 tagged *O*-CMC-metformin NPs was studied in MiaPaCa-2 and L929 cells after 24 h incubation (Fig. 5). Cells were also counter stained with DAPI, to identify intracellular localization of NPs. The untreated control cells showed only DAPI fluorescence while cells treated with NPs showed green fluorescence of the rhodamine tagged NPs along with DAPI fluorescence. The appearance of green fluorescent spots at higher magnification clearly established internalization of NPs by the cells. Both L929 and MiaPaCa-2 cells showed green fluorescence, which indicated nonspecific uptake of the NPs by normal as well as pancreatic cancer cells.

## 4. Conclusion

Metformin loaded *O*-CMC NPs were successfully prepared by ionic gelation method to reduce its solubility in aqueous medium. The prepared NPs were analyzed using DLS and SEM for its size and shape. The FT-IR analysis confirmed the incorporation of the drug within the nanocarrier. The developed nanoformulation showed burst drug release followed by slow and sustained release of the drug at neutral pH and hence may increase the drug retention time in blood/circulation. The *O*-CMC metformin nanoparticles did not induce any hemolysis and thus proved to be hemocompatible. Even though the NPs showed nonspecific uptake by both normal and cancer cells, they presented a dose dependent preferential toxicity toward pancreatic cancer cells. The preferential toxicity of *O*-CMC-metformin NPs toward pancreatic cancer cells could be due to metformin-mediated inhibition of mTOR activity in pancreatic cancer cells. Hence by the application of this nanoformulation, bioavailability of metformin can be increased without losing its anticancer property for efficient treatment of pancreatic cancer as well as type 2 diabetes.

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