World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers
Available online at: http://www.wjpsonline.org/
Original Article

Carboxymethyl cellulose based multifunctional targeted drug delivery platform for pancreatic cancer: Nanotheranostic potential and biocompatibility analysis

Preeti Nigam Joshi*, Shishanka Wangnoo, Michelle Louis

Combichem Bioresource Centre, National Chemical laboratory, Pune, 411008, India

Received: 04-03-2015 / Revised: 06-06-2015 / Accepted: 25-06-2015

ABSTRACT

Nanotechnology has exhibited immense potential in the fields of medical and biotechnology with its notable intervention from disease diagnostics to therapy. Cancer nanotechnology is an emerging research arena significantly benefited by the advancements of nanotechnology as it provides potential alternative to conventional chemotherapy with active ligand receptor targeting, specific to cancer cells only that reduces the harmful side effects of traditional chemotherapy. The basic objectives of this study was to provide a new insight for pancreatic cancer specific drug delivery by exploring quercetin loaded hyaluronic acid (HA) coupled carboxymethyl cellulose nanoparticles (CMC) based theranostic system for cancer treatment and imaging. Pancreatic adenocarcinoma is considered to be the most difficult malignancy to handle and it also possesses the worst mortality rate amongst all cancers. In most of the cases it is diagnosed in later stages with limited treatment options. Moreover pancreatic cancer is resistant to conventional chemotherapy that restricts the effectiveness of the treatment. In this regard it is indispensable to develop treatment strategies to optimize drug delivery for pancreatic cancer to improve the prognosis of patients with this malignancy. Based on our experimental findings it can be said that our Quercetin loaded CMC-NPs can be developed as an effective antineoplastic nanovector for the treatment of this fatal disease. Apart from the anticancer efficacy, we exploited the florescence properties of quercetin in bioimaging analysis as well.

Key words: Quercetin, Pancreatic cancer, Drug Delivery, Nanotheranostics, Carboxymethyl cellulose, Polymer nanoparticles

INTRODUCTION

Nanotechnology is undoubtedly the most distinguished research arena in recent times that has been deeply influenced various scientific fields including medicine and diagnostics. The enormous applications of nanoparticles in medical field have given rise to a new branch of nanomedicine or medical nanotechnology. Nanoparticles can be tuned to possess different properties and release characteristics for the best delivery or encapsulation of the therapeutic agent based on the method of preparation. The tissue specific selectivity and ability of nanovectors as drug carriers to overcome biological barriers has revolutionized the field of oncology because apart from providing long circulation half-lives of

***Corresponding Author Address:** Preeti Nigam Joshi, Combichem Bioresource Centre, National Chemical laboratory, Pune, 411008, India*

drugs with superior pharmacokinetic properties, nanoformulations with a targeted pendant group for specific cell surface receptor allow higher dose to be delivered to the target site with diminished side effects of conventional chemotherapy. [1-3] Nanotheranostic is a new term coined to couple the targeting and imaging properties of a nanocarrier for image guided drug delivery.

In this study, for the first time we have reported the synthesis of quercetin loaded carboxymethyl cellulose (CMC) nanoparticles functionalized with hyaluronic acid (HA) as a nanotheranostic system specific for pancreatic cancer therapy and diagnosis. Pancreatic adenocarcinoma is the most fatal cancer with the worst mortality rate among all cancers. It is the fourth leading cause of cancer related deaths in the United States and eighth worldwide due to its extremely poor prognosis in locally advanced or metastasized stage. [4, 5] Its aggressive metastasis makes it resistant to most of the conventional therapies that exhibit only palliative effect with minimal impact on survival. Due to the enriched tumor stromal component and disorganized vasculature of pancreatic cancer tissues, efficient delivery of therapeutic agents is quite difficult [6-8] that makes it crucial to discover innovative targeted drug delivery strategies to treat this lethal disease. Though many nano-delivery systems have already been reported for different cancers but very few reports are there with pancreatic cancer specific treatment strategies.

In this study the prime objectives were to develop a simple yet effective targeted nanotheranostic system based on CMC for this deadliest cancer and to estimate the biocompatibility and efficacy of the system. Polymeric nanoparticles made from natural or synthetic polymers have attracted a lot of interest due to their easy surface modification properties and stability. Among natural polymers, cellulose and its corresponding neutral, acidic and basic derivatives have a long history as pharmaceutical excipients in various types of formulations.

The presence of hydrophilic reactive groups like amino, carboxylic and hydroxyl on natural polymers significantly enhance their surface adhesion property apart from providing surface modification capabilities. Carboxymethyl cellulose is a water soluble cellulose derivative with carboxymethyl groups (-CH2COOH) bound to some of the OH groups of the glucopyranose monomers that make up the cellulose backbone. Owing to its viscosity, biodegradability, biocompatibility, non-toxicity, hypoallergenic nature, cost effectiveness and availability in wide forms, carboxymethyl cellulose has immense applications in pharmaceutical area. [9, 10] Although some applications of CMC in drug delivery have already been reported [9, 11, 12] but the present study is a pioneer combination of quercetin loaded for pancreatic cancer specific bioimaging and drug delivery.

The surface modified CMC-NPs exhibited improved bioavailability of the encapsulated drug to the target site. Quercetin ((3,3',4',5,7-pentahydroxyflavone) is a dietary polyphenol commonly found in apples, onions, green tea etc and is widely used in health care products. Apart from its industrial applications, quercetin has well proven anticancer activity also. [13, 14] Different mechanisms of action of quercetin have been revealed earlier by many researchers. It induces TNF related apoptosis in cancer cells by increasing the expression of death receptor. [15] Vijayababu et. al. have reported that quercetin also induces cell cycle arrest at G2/M or G1/S transitions by modulating cyclin B and topoisomerase. [16]

Quercetin can also target PI3K-Akt/PKB pathways in different cells, promoting cancer cell apoptosis and inhibiting cancer cell proliferation and tumor genesis. [17, 18] Irrespective of the potent anti-cancerous effect of quercetin with wide spectrum of pharmacological properties, this molecule has limited applicability due to its instability in physiological medium and low water solubility as these properties are responsible for its poor bioavailability and extensive first pass clearance before reaching the site of action.[19, 20]

In this regard while keeping in mind the potential of QC in cancer therapy, we put forth a new approach to increase its bioavailability by loading it onto functionalized CMC-NPs. In few previous reports QC was loaded into chitosan and Lecithin nanoparticles [21, 22] for stability enhancement but to the best of our knowledge, this is the first report on application of QC for active tumor targeting. CMC-NPs were functionalized with HA to enhance the theranostic applicability of QC-CMC-NPs for pancreatic cancer specific treatment and imaging. Hong et. al. found out that CD-44 positive cells are responsible for drug resistance in pancreatic cancer cells. CD-44 is a pancreatic cancer stem cell surface marker which is highly expressed in human pancreatic adenocarcinoma cells and is responsible for proliferation and reconstitution of resistant pancreatic cancer cells. [23]

As a remedial application, targeted therapy against CD-44 cells can overcome drug resistance in the treatment of pancreatic cancer. Hyaluronic acid (HA) is a natural polysaccharide and a major component of the extracellular matrix and synovial fluids of the body. It binds to various cancer cells that over-express CD44, an HA receptor. [23, 24] We utilized HA as targeting moiety of the CMC- Quercetin conjugates for pancreatic cancer therapy. Another important aspect of a nanotheranostic system is bio-imaging or visualization of living cells that is very crucial for medical diagnostic purpose. Previous studies have reported the ample use of fluorescent agents (rhodamine, fluorescein) for this purpose.

Apart from the fluorescent dyes, semiconductor quantum dots (made up of semiconductor materials i.e. cadmium, zinc, selenium) have been massively exploited as cell illuminators due to their excellent and stable fluorescence [9, 25] but the inherent toxicity and high cost associated with cadmium/selenium QDs are cause of concern that restrains their biological application. [25, 26] For this reason there is a need to find better alternatives for bio-imaging and visualization. As earlier reported by Baran et. al., QC exhibits auto fluorescence property like curcumin so we tried to explore this special trait for visualization of QC uptake by pancreatic cancer cells *in vitro*. [27] In this analysis, we have evaluated the biocompatibility and theranostics potential of the multifunctional CMC nanoformulation for pancreatic cancer specific therapy. To the best of our knowledge, no HA conjugated and quercetin loaded CMC based nanotheranostic system has been reported yet.

MATERIALS AND EXPERIMENTAL DETAILS

Chemicals: Carboxy methyl cellulose (CMC), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), Quercetin, Dimethyl Sulfoxide (DMSO), MTT (3-[4, 5-dimethylthiazol-2-yl]-3, 5-diphenyltetrazolium bromide) and Hyaluronic acid (HA) were obtained from Sigma-Aldrich and used as received. CD-44 expressing pancreatic cancer cell line

(Panc-1) was procured from National Centre from Cell Science (NCCS-India) Cell Repository and was cultured in DMEM media (Himedia- India) supplemented with 10 % FBS (Gibco, Life Technologies, India).

Synthesis of CMC nanoparticles: The CMC nanoparticles were synthesized by single emulsification method. This system consisted of two phases i.e. an organic phase and an aqueous phase. The organic phase was a mixture of dichloromethane (DCM) and acetone in a ratio of 60:40 while the aqueous phase was formed by 2.5 % CMC solution in deionized water. The organic phase was then dispersed in the aqueous phase drop by drop during sonication at 63^0C. The CMC nanoparticles that were formed by this method were separated by centrifugation at 13500 rpm for 5 minutes and washed thrice with deionized water. After separation, the size distribution and morphology of the final nanoparticles was examined by SEM and DLS. To prepare drug loaded nanoformulation, quercetin was encapsulated and surface adsorbed on CMC-NPs. In brief 25 mg/ml of quercetin (1:20 DMSO: water) was mixed with the solution of CMC prior to the addition of organic phase so that it could be encapsulated into the nanovesicles. After synthesis, the drug loaded nanoparticles were centrifuged and resuspended into a Quercetin solution of 20 mg/ml and kept on magnetic stirring for 6 hrs at room temperature. The resulting solution was centrifuged and collected pellet was kept at 4^0 C for further use. CMC-NPs were functionalized with HA via DCC-DMAP coupling of carboxylic group on HA and hydroxyl group of CMC.

Characterization

Nanoparticles size and morphology: SEM (Quanta 2003D, FEI, The Netherlands), was performed to identify the morphological details of CMC and QC-CMC nanoparticles**.** A diluted sample of nanoparticles was drop cast on silicon wafers and dried properly for observation.

UV-Vis Spectroscopy: UV-Vis spectroscopy (Cary50 Bio, Varian, The Netherlands), was done to obtain the absorption spectra of NPs in the range of 200-800 nm. Samples were measured in I cm path length quartz cuvette at 25^0 C. All the experiments were performed in triplicate.

Fourier Transformed Infrared Spectroscopy (FT-IR): FT-IR spectra of lyophilized samples of CMC and HA-CMC and was taken by FT-IR spectrometer (Nicolet iS5, Thermo scientific, USA). The samples we converted into 1 mm pallets with KBr and measured in the range of 500-4000 cm^{-1}.

Photoluminescence Spectra of QC and CMC loaded QC: Photo Luminescence (LS 55, Perkin Elmer, USA) spectroscopy was performed to observe the variation in fluorescence properties of QC after encapsulation in CMC-NPs between 250-650 nm. The emission spectrum was taken at 440 nm excitation wavelength.

Drug encapsulation and drug release profile: Quercetin encapsulation and release profile was established via UV-Vis spectroscopy. The Quercetin loaded HA conjugated CMC-NPs were centrifuged at 12,500 rpm for 20 min. The collected pellet was washed twice and resuspended in deionized water. After freeze drying the powdered sample was stored at 4^0 C for further use. The supernatant having unencapsulated drug was collected separately and absorbance was measured at 375 nm. Based on the standard curve of Quercetin, free drug concentration was calculated. Encapsulation efficiency (EE %) was calculated from the following formula:

$$EE\ (\%) = [(C_{TD} - C_{FD}) / C_{FD}] \times 100$$

Where C_{TD} is concentration of total drug and C_{FD} is concentration of drug in supernatant.

In vitro drug release profile of Quercetin from nanoparticles was carried out in PBS buffer (pH 7.4). 1.0 mg nanoparticles were dispersed in 25 ml of PBS and were placed in shaker-incubator at 37°C 100 rpm. To estimate the release % of Quercetin, at predefined time intervals of 2 hrs, 3 ml of supernatant from the sample was withdrawn and absorbance was recorded at 373 nm. After sample removal, the suspension was refilled with 3 ml fresh PBS.

Quercetin % release = C_{DS} / C_{TD} X100

Where, C_{DS} is the concentration of Quercetin released in the suspension and C_{TD} is the total amount of Quercetin encapsulated in the nanoparticles.

***In vitro* cytotoxicity and Imaging analysis:** MTT (3-[4, 5-dimethylthiazol-2-yl]-3, 5-diphenyltetrazolium bromide) assay was performed to analyze the efficacy of our nanoformulation. Panc-1 (pancreatic cancer cell line) was incubated in DMEM media with 10% FBS and 50 µL gentacyin at 37^0 C in 5 % CO2 environment. Cells were seeded in 96 well plates for 24 hrs and CMC-NPs, CMC-HA, CMC-Quercetin, CMC-Quercetin-HA and free Quercetin with concentration of 5, 10, 25, 50 and 100µg/ml was added to cells and further incubated for 24 hrs into the same environment. After second incubation, MTT (10 µl, 10mg/ml) was added and kept for 4 hrs. Subsequently, culture media was removed and DMSO (150 µl) was added and after 15 minutes incubation with shaking, optical density (OD) was taken at 570 nm. All the experiments were performed in triplicate to avoid experimental errors. For imaging, QC-HA-CMCs were injected into the Panc-1 (Pancreatic cancer cell line) and incubated for 6 h. Panc-1 cell lines were maintained in DMEM media supplemented with 10% fetal bovine serum in 5% CO2 atmosphere at 37^0 C. Cellular uptake of nanoformulation was visualized by HCS system (Array Scan, Thermo Scientific, USA).

RESULTS & DISCUSSION

The major aim of this study was to synthesize a biocompatible and efficient theranostic system specifically for pancreatic cancer and we introduced a new drug delivery system based on HA conjugated and quercetin loaded fluorescent CMC nanoparticles for cancer cells imaging and targeting. Chemotherapy; the most preferred form of cancer treatment suffers from major side effects and this is the reason new strategies are being developed for effective yet less harmful mode of treatments for this deadliest disease. In this analysis we emphasized on designing a theranostic system specifically for pancreatic cancer and checked the efficacy of our system *in vitro*. The prime concern for targeting pancreatic cancer was its late prognosis and high mortality rate. Moreover very few reports specifically focused on developing targeted drug delivery system for this most fatal cancer and ours is the first report of carboxymethyl cellulose based nanoformulation for pancreatic cancer. CMC owing to its excellent biocompatibility and cost effectiveness has vast industrial applications. In this analysis we have explored the CMC nanoparticles as an efficient nano drug delivery vehicle. Carboxymethyl cellulose nanoparticles have been prepared by simple single water in oil (w/o) emulsification method. [28] This technique has been widely explored for other polymers and polysaccharides [29-31] but this the first report for CMC nanoparticle synthesis using this method. CMC was dissolved in double distilled water while organic phase consist of DCM and acetone (ratio of DCM: acetone was

optimized to 6: 4 for the synthesis of smaller nanoparticles) and spherical nanoparticles of size range 100-180 nm were synthesized via this method.

Figure 1 represents the SEM of CMC-NPs. The hydrodynamic distribution obtained from DLS analysis is exhibited in **Figure S1** of electronic supplementary material. In previous reports folic acid conjugated CMC-NPs have been synthesized and applied for targeted drug delivery [9, 32] but folic acid receptor is present on healthy cells as well, that limits the utility of FA conjugated systems as an efficient targeted delivery systems. So it is imperative to depend on other cell surface receptors more specific for cancerous cells only, for active receptor-ligand targeting strategies. In the present study, we took advantage of the free carboxylic groups on the surface of CMC-NPs to functionalize it with hyaluronic acid. HA is a naturally occurring polysaccharide and is the prime endogenous ligand for CD44 (a cancer stem cell marker) and cell surface receptor over expressed on a variety of cancer cells specifically. It also plays an important role in cancer development, metastasis and in the interaction between cancer cells and extracellular matrix. Pancreatic cancer cells are rich in CD44 receptors which are responsible for the chemotherapy resistance and recurrence of cancer after treatment.

In this regard, a delivery system based on HA conjugated nanoparticles can be very effective against drug resistant pancreatic cancer and may enhance the life expectancy in this fatal disease. CMC nanoparticles were successfully functionalized with HA via DCC-DMAP coupling by conjugating the –COOH group of CMC to –OH group of HA. TGA analysis revealed that approximately 22 % weight loss occurred as evidenced by the appearance of a new step in HA functionalized CMC-NPs **(Figure 2)** which indicated the successful functionalization of CMC with hyaluronic acid. The variations in FT-IR spectra of CMC, CMC-HA also supported the conjugation of HA. The peak at 1740 cm^{-1} represents the C=O ester bond stretching in HA conjugated CMC-NPs **(Figure S2).** Zeta potential of blank CMC-NPs and HA-CMC-NPs was determined via dynamic light scattering (DLS) and found to be -27.23 and -32.58 mV respectively as exhibited in **Figure S3.** HA is negatively charged at physiological pH as indicated from the zeta potential analysis. A substantial surface charge indicates effective water suspension and is advantageous for storage and administration of nanoparticles. Apart from decorating CMC with HA for active drug delivery, efforts were taken to develop the nanoformulation as a complete nanotheranostic system by attaching fluorescent molecule for bioimaging and we took advantage of the fluorescent properties of Quercetin. In this study we tried to explore this property to estimate cellular uptake and cancer cell imaging by monitoring its cellular internalization via HA mediated endocytosis.

Though traditionally organic dyes and quantum dots due to their photoluminescence have been widely applied in bioimaging applications, this is the first report of application of QC in cancer cell imaging. Spectroscopic evaluation of free QC and CMC-QC was done to confirm the encapsulation of QC into CMC-NPs. The UV-Vis spectrum of free QC exhibited two characteristic peaks at ~260nm and ~375 nm described previously. Though QC-CMC nano-complex also showed two peaks but the intensity was reduced that can be attributed to its encapsulation in CMC-NPs **(Figure 3).**

For photoluminescence studies, QC was encapsulated within CMC-NPs and

fluorescent spectrum was taken at excitation wavelength of 440 nm. It showed a substantial increase in intensity **(Figure 4)** as observed by Zhang et. al. earlier. The possible cause of this enhancement may be the more protected microenvironment of nanoparticles that prevents the active fluorophore from environmental decays. [33, 34] Moreover the difference in FT-IR spectra of free quercetin and CMC encapsulated quercetin also supported the successful encapsulation of QC in CMC-NPs. **(Figure S4)**

For drug encapsulation studies and release profile estimation, UV spectroscopy was performed in phosphate buffer (pH 7.0) (**Figure 5**). Figure S5 revealed that QC showed approximately 46 hrs of sustained release. The bioimaging analysis of Panc-1 cells revealed the effective internalization of QC-CMC-NPs into the cells as evidenced from the strong green fluorescence inside the cells resulting from the efficient uptake of HA-CMC-NPs by Panc-1 cancer cells as shown in **Figure 6(c).** On the other hand, unfunctionalized CMC-NPs exhibited weak fluorescence signal, indicating that a lesser amount of nanoparticles in comparison to HA-CMC-NPs are internalized into the cancer cells due to absence of the target moiety for CD-44 positive Panc-1 cells as exhibited in **Figure 6(b).**

It is clearly evident that HA-CMC-NPs can efficiently target CD-44 over expressing pancreatic cells via HA mediated endocytosis pathway. QC is a slightly fluorescent molecule but its encapsulation in CMC-NPs not only averted its disintegration into the cells but also provided a significant stable fluorescent that the cellular uptake of QC can easily be predicted by its own fluorescence that in turn eliminates the need of separate bioimaging agent.

After confirming the cellular uptake of nanoparticles, the anti-cancerous efficacy of nanoformulations was analyzed *in-vitro* via MTT assay. Panc-1 cells were incubated with CMC-Quercetin, CMC-HA-Quercetin and free Quercetin.

As shown in **Figure S6,** HA-CMC-Quercetin formulation was the most effective one due its targeted internalization into CD-44 expressing Panc-1 cells. From the MTT data it can be concluded that CMC and HA-CMC nanoparticles were quiet biocompatible and showed almost no toxicity to Panc-1 cells with excellent cell viability 89-85 % respectively at different concentrations (50-200 µg/ml). The data obtained from the MTT assay demonstrated that the cytotoxicity was rendered by the encapsulated drug alone as the native nanoparticles were quite biocompatible. The IC50 value for the free QC was 14.93µg/ml that got reduced to 9.5µg/ml after its encapsulation in HA-CMC-NPs, indicating its better bioavailability after loading to Panc-1 cells as indicated in **figure. 7**. It can be concluded that our hyaluronic acid modified CMC-NPs has a theranostic potential to be an efficient nano-vector for treatment of pancreatic cancer, the deadliest in all cancers.

Chemotherapy; the most preferred form of cancer treatment suffers from major side effects and this is the reason new strategies are being developed for effective yet less harmful mode of treatments for this deadliest disease. In this analysis we emphasized on designing a theranostic system specifically for pancreatic cancer and checked the efficacy of our system *in vitro*. The prime concern for targeting pancreatic cancer was its late prognosis and high mortality rate. Moreover very few reports specifically focused on developing targeted drug delivery system for this most fatal cancer and ours is the first report of carboxymethyl cellulose

based nanoformulation for pancreatic cancer. CMC owing to its excellent biocompatibility and cost effectiveness has vast industrial applications. In this analysis we have explored the CMC nanoparticles as an efficient nano drug delivery vehicle. CMC-NPs were synthesized via simple w/o emulsion method and spherical nanoparticles of sizes less than 200 nm were obtained. Morphological characteristics like size, polydispersity index deeply influence loading and release characteristics of the active molecule inside the nanoparticles are few prime factors that affect the efficacy of a theranostic system. Smaller nanoparticles with low dispersity index are considered ideal vectors due to their stability, higher surface area and better sustained released properties. Nanospheres exhibit chemotherapeutic advantages of biodegradability and enhanced permeability and retention effect (EPR) for cancer therapy. [35] Thus, these are the most applied form of drug delivery vector. Earlier studies have also shown that phagocytosis of tumor cells depends on size of nanoparticles and nanoparticles with 100-200 nm diameters preferentially accumulate on tumor cells than healthy cells. [36]

Though Gemcitabine is the most preferred drug for pancreatic cancer treatment but like other chemotherapy drugs, it also leaves enormous side effects. In recent years, researchers from all over the world are trying to identifying new molecules with potent anticancer efficacy. In this regard natural compounds from plants and other sources with less or no side effects can be developed as an efficient substitute of chemical anticancerous agents. In this study we have estimated the potential of quercetin for pancreatic cancer therapy as well as imaging; the most distinguished feature of this work where the fluorescence of active drug itself was exploited to determine its cellular internalization. Quercetin due to its enormous health benefits containing antioxidant, anti-cancerous and anti-inflammatory actions have vast applicability.

The antioxidant properties and cancer preventive properties have previously been reported but there wasn't any evidence of determining its potential in pancreatic cancer treatment via active targeting. Moreover it also exhibits fluorescence and although these properties vary with pH and temperature, CMC-NPs encapsulated QC showed improved characteristics in terms of stability and luminescence as exhibited in **figure 4**. A clearly distinguished FT-IR spectra of CMC loaded quercetin and free QC indicated the successful encapsulation of drug into the nanoparticles.

Though there are many drug delivery systems have been proposed but many of them suffers from the drawback of nonspecific mode of delivery so in our case we have functionalized the CMC-NPs with hyaluronic acid that is the targeted moiety for CD-44 receptor overexpressed on drug resistant pancreatic cancer cells. HA functionalized Quercetin-CMC nanoformulation is more advantageous over other systems because of its specific targeting towards pancreatic cancer, sustained release of quercetin and low toxicity to healthy cells. HA is also negatively charged at physiological pH that is also an added advantage as previously reported, anionic nanocarriers are more preferred for targeted drug delivery than their cationic counterparts due to nonspecific binding of cationic vehicles to different cells after systemic administration. Moreover electrostatic repulsion among negatively charged nano-spheres also prevents their aggregation to evade inactivity. [37, 38] The negative zeta potential (**Figure S3**) of the nanoformulation and the stability of

nanoformulation supported the above hypothesis.

It can be concluded by considering these facts that our quercetin loaded nanoformulation has potential to be developed as an efficient drug delivery tool.

As earlier reported by us for drug loading, in this analysis also we have amalgamated both the techniques (direct drug loading and physical adsorption at the surface of nanoparticles) to prepare nanoformulation with better encapsulation efficiency and release profile. Direct loading (encapsulation of drug) and mere physical absorption on nanoparticles possess their own benefits and shortcomings i.e. direct loading suffers from the disadvantage of low encapsulation and loading efficiency in comparison to physical adsorption, but exhibits better sustain release properties than the latter one. As discussed earlier UV analysis revealed that approximately 85% drug encapsulation efficiency was achieved via above described method **(figure 5).** All the experiments were performed in triplicate and the average data values are presented. The *in vitro* release profile of QC showed ~46 hrs sustained release at 37 ^{0} C in PBS (**Figure S5**) with a biphasic release pattern with initial burst release followed by a slow release pattern up to ~46 hrs, the mechanism of which can be explained by drug leaching or detachment in the first phase of fast release due to physically adsorbed drug on the surface of nanoparticles while first order diffusion controlled behavior was responsible for slow release of the drug.

In recent years, significant progress have been made in search of new cell imaging agents that could meet two main criteria of biocompatibility and stability. Though semiconductor quantum dots seems promising due to their stable fluorescence but biocompatibility is a big issue associated with them while organic dyes are biocompatible and economic but suffers from small shell life. In this regard, the approach demonstrated by us is an entirely new way of cell imaging where we explored the possibility of utilizing the fluorescence of active drug quercetin for determining the cellular uptake of drug molecules by its own fluorescent properties. The NPs having HA targeting moiety exhibited significant fluoresce in pancreatic cells as demonstrated in **fig. 6.** The diminished fluorescence in fig 6(b) is due to CMC-NPs without HA functionalization, which itself explains that the functionalized NPs are internalized into the cells due to HA mediated endocytosis and responsible for the better illuminated images of cells (Fig 6(c)) than fig. 6b. The results exhibited in figure 6 are significant to prove that in comparison to free quercetin (where no fluorescence was observed due to its feeble and unstable photoluminescence properties), HA-CMC-NPs loaded quercetin showed improved bioavailability to cancer cells due to the effective internalization and cellular uptake of functionalized nanoparticles.

In native state quercetin exhibits less bioavailability due to is insolubility in aqueous medium but after encapsulating it into HA functionalized CMC-NPs, a significant increase in terms of bioavailability was obtained as evidenced from the results of MTT assay where less IC50 (~10 µg/ml in comparison to ~15 µg/ml free QC) concentration was required to get the desired antiproliferative efficacy of quercetin. Moreover the extremely sensitive photoluminescence profile also showed improvisation in terms of intensity and stability after loading in CMC-NPs as exhibited in **figure 7**.

This clearly indicates that the nanotheranostic system reported in this analysis has immense potential to be

developed as an efficient tool for pancreatic cancer diagnosis and therapy. Encapsulation of quercetin in CMC-NPs not only enhanced and stabilized its week fluorescence but also significantly increased the bioavailability that is a major breakthrough for its therapeutic potential. It is the first repot of targeted delivery system for quercetin and another important advantage elaborated by our theranostic system is ease and simplicity of synthesis procedures where without indulging in tedious protocols and methodologies as previously reported with many polymeric drug delivery systems, we designed a simple, cost- effective and biocompatible nano-platform for cancer diagnostic and therapy.

CONCLUSION

The present study is a collection of data facts from the nanoformulation comprised of quercetin loaded CMC-NPs decorated with HA and deals with its theranostic potential for drug resistant pancreatic cancer treatment. The results obtained were very encouraging with respect to the sustained release profile of quercetin, HA mediated active targeting and bioimaging abilities exhibited by quercetin itself. Pancreatic cancer is the deadliest one with high mortality rate that makes it imperative to design new therapy modules for its treatment. This study also shows that natural medicinal compounds with tailored properties have the potential to be developed as efficient alternatives to the conventional therapies. This study is a novel example of quercetin loaded functionalized CMC-NPs with very promising results on pancreatic cancer cell lines and studies are going on for further improvisation of the nanoformulation.

Acknowledgement

The authors are grateful to the Department of Science of Technology, Government of India, for providing the necessary funding to meet the research expenses. We express our sincere thanks to Dr. Satish B. Ogale, Chief Scientist, and Dr. Dhiman Sarkar, Senior Principal Scientist, National Chemical Laboratory for their help and support.

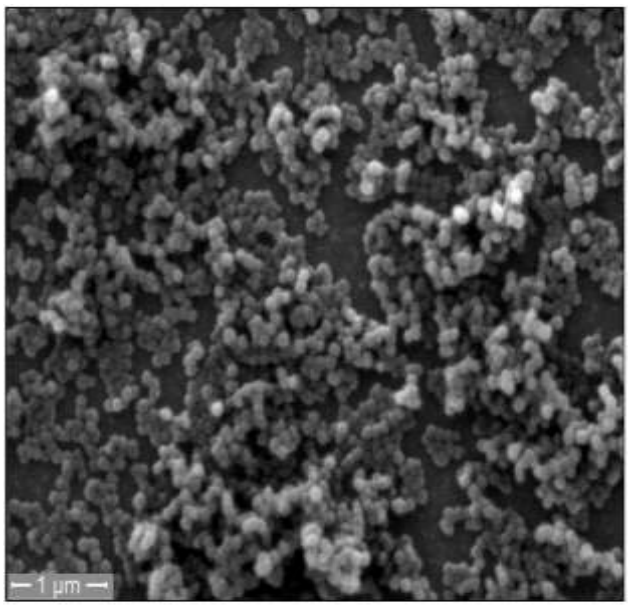


Figure 1: SEM image of CMC nanoparticles. Average particle size ~100-180 nm.

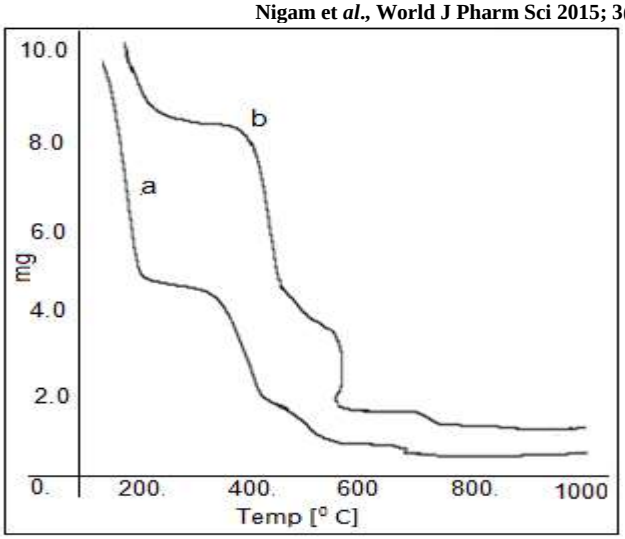


Figure 2: TGA curves for a) CMC nanoparticles; b) HA functionalized CMC-NPs (Ramp rate: 10^0C/min).

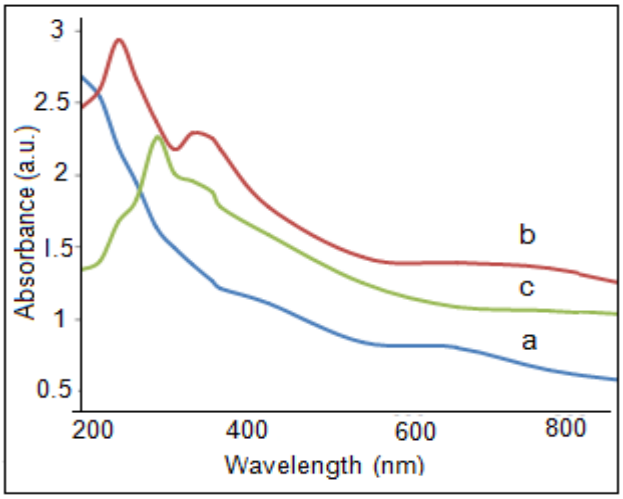


Figure 3: UV Vis spectra of a) CMC-NPs; b) free QC; c) CMC encapsulated QC. (All the samples were prepared in DI water and experiments were performed at room temperature ~25 ^{0}C).

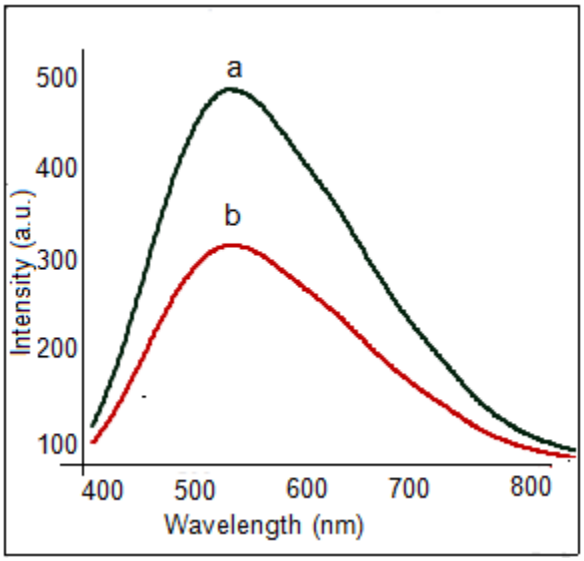
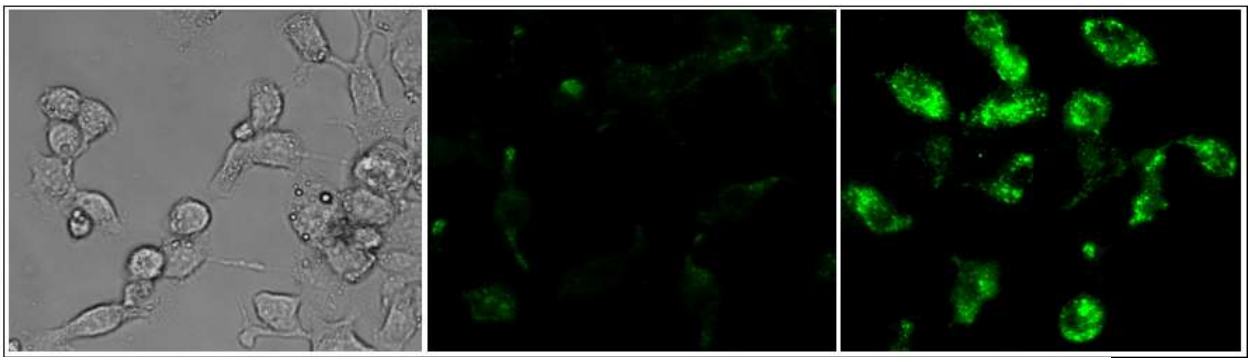


Figure 4: Photoluminescence spectra of a) CMC encapsulated QC; b) Free QC. (Excitation wavelength: 440 nm).



a b c

Figure 5: Fluorescence imaging of Panc-1 cells after 24 hrs incubation in presence of quercetin loaded CMC-NPs in DMEM media with 10 % FBS in CO_2 incubator at 37 ^{0}C; (a) Cells without staining (control), (b) QC-CMC-NPs without HA functionalization, (c) QC-CMC-NPs functionalized with HA.

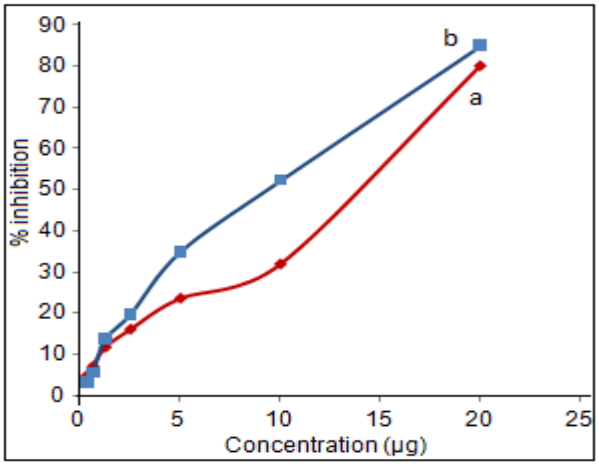


Figure 6: Dose response curve of a) Free QC solution; b) CMC-HA-QC nanoparticles

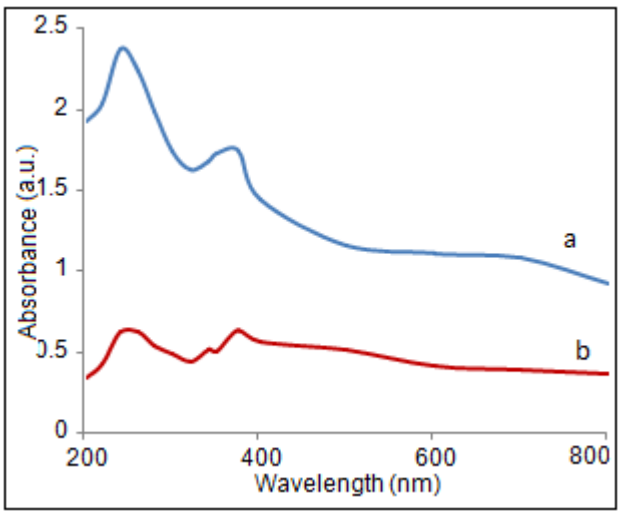


Figure 7: UV-Vis spectrum of aqueous solution of free QC (5 mg/ml) (a) and (b) supernatant of QC loaded CMC NPs at room temperature (~25 ^{0} C).

REFERENCES

1. Ambede AV et al. Dendrimeric micelles for controlled drug release and targeted delivery. Mol Pharm 2005; 2: 264-72.
2. Chen H et al. Thermal responsive micelles for dual tumor-targeting imaging and therapy. Nanoscale 2013; 5: 12409-424.
3. Wei X et al. Retro-Inverso Isomer of Angiopep-2: A Stable D‑Peptide Ligand Inspires Brain-Targeted Drug Delivery. Mol Pharm 2014; DOI: 10.1021/mp500086e.
4. Yachida S, Iacobuzio-Donahue CA. The pathology and genetics of metastatic pancreatic cancer. Arch Pathol Lab Med 2009; 133(5), 413-22.
5. Papa AL et al. Mechanistic studies of gemcitabine-loaded nanoplatforms in resistant pancreatic cancer cells. BMC Cancer 2012; 12: 419-28.
6. Kalluri, Zeisberg R. Fibroblasts in cancer. Nat Rev Cancer 2006; 6(5): 392-401.
7. Hwang RF, Moore T, Arumugam, Ramachandran T, Amos V, Rivera KD, Ji A, Evans DB, Logsdon CD. Cancer-associated stromal fibroblasts promote pancreatic tumor progression. *Cancer Res*. 2008: 68(3): 918-926.
8. Lee GY et al. Theranostic nanoparticles with controlled release of for targeted therapy and MRI of pancreatic cancer**.** ACS Nano 2013; 7: 2078-89.
9. Aswathy RG et al. Multifunctional Biocompatible Fluorescent Carboxymethyl Cellulose Nanoparticles. J Biomater Nanobiotechnol 2012; 3: 254-60.
10. Biswal DR, Singh RP. Characterization of carboxymethyl cellulose and polyacrylamide graft copolymer. Carbohydr Polym 2004; 57(4): 379-87.
11 Mathew ME et al. In Vitro and In Vivo Evaluation of pH-Sensitive Hydrogels of Carboxymethyl Chitosan for Intestinal Delivery of Theophylline. Carbohydr Polym 2010; 80: 442-48.
12. Ojha M et al. Synthesis and evaluation of sodium carboxymethyl cellulose azo polymer for colon specificity. Int Curr Pharm J 2012; 1: 209-12.
13. Zhu S et al. Strongly green photoluminescent graphene quantum dots for bioimaging applications. Chem Commun 2011; 47: 6858-60
14. Son HL, Anh, NP. Phytochemical composition, in vitro antioxidant, anticancer properties of quercetin from methanol extract of Asparagus cochinchinensus (LOUR.) Merr. Tuber. J Med Plant Res 2013; 7: 3360-66.
15. Jung YH et al. Quercetin enhances TRAIL-induced apoptosis in prostate cancer cells via increased protein stability of death receptor 5. Life Sci 2010; 86: 351-57.
16. Vijayababu MR et al. Quercetin downregulates matrix metalloproteinases 2 and 9 proteins expression in prostate cancer cells (PC-3) J Mol Cell Biochem 2006; 287: 109-16.
17. Fresno Vara JA et al. PI3K/Akt signalling pathway and cancer. Cancer Treat Rev 2004; 30: 193-04.
18. Chen J. Multiple signal pathways in obesity-associated cancer. Obes Rev 2011; 12(12): 1063-70.
19. Kumari A et al. Development of biodegradable nanoparticles for delivery of quercetin Colloids and Surfaces B: Biointerfacesn 2010; 80: 184–92.
20. Ratnam DV et al. Role of antioxidants in prophylaxis and therapy: A pharmaceutical perspective. J Control Release 2006; 113: 189–07.
21. Souza MP et al. Quercetin-Loaded Lecithin/Chitosan Nanoparticles for Functional Food Applications. Food Bioprocess Technol 2014; 7: 1149–59.
22. Zhang Y et al. Physicochemical characterization and antioxidant activity of quercetin-loaded chitosan nanoparticles. J App Poly Sci 2008; 107: 891–97.
23. Hong SP et al. CD44-positive cells are responsible for resistance in pancreatic cancer cells. Int J Cancer 2009; 125: 2323-31.
24. Platt VM, Szoka FC. Anticancer therapeutics: targeting macromolecules and nanocarriers to hyaluronan or CD44, a hyaluronan receptor. Mol Pharm 2008; 5(4): 474-486.
25. Kairdolf BA et al. Semiconductor Quantum Dots for Bioimaging and Biodiagnostic Applications. Annu Rev Anal Chem (Palo Alto Calif). 2013; 6: 143-162.
26. Chen N et al. The cytotoxicity of cadmium-based quantum dots. Biomaterials. 2012; 33: 1238-1244.
27. Barani I et al. Fluorscent properties of quercetin in human leukemia jurket T cells. Rom. Journ. Phys. 2011; 56: 388–98.
28 Kumar SG, Aminabhavi TM. Synthesis and characterization of modified chitosan microspheres: Effect of the grafting ratio on the controlled release of nifedipine through microspheres. J Appl Polym Sci 2003; 89 (11): 2940-49.
29 Gaudana R et al. Encapsulation of Protein-Polysaccharide HIP Complex in Polymeric Nanoparticles. J Drug Deliver 2011; doi:10.1155/2011/458128.
30. Chin SF et al. Synthesis and characterization of modified chitosan microspheres: Effect of the grafting ratio on the controlled release of nifedipine through microspheres. J Nanomat. 2014; 2014: 1-7
31. Alam S et al. Development and evalution of thymoquinone loaded chitosan nanoparticles for nose to brain targeting: a pharmoscintigraphic study. Int J NanoMed 2012; 7: 5705-18.
32. Zhao X et al. Emerging application of quantum dots for drug delivery and therapy Expert Opin Drug Deliv 2008; 5: 309-19.
33. Zhu L et al. Comparative study of fluorescence enhancement of some fluorsence system in β-cyclodextrin derivatives and β-cyclodextrin surfactant media. Microchem. J 1996; 53: 361-70.
34. Petros RA, DeSimone JM, Strategies in the design of nanoparticles for therapeutic applications. Nat Rev Drug Discover 2010; 9: 615-27.
36. Bae YH, Park K. Targeted drug delivery to tumors: myths, reality and possibility. J. Controlled Release 2012; 153(3): 198-05.
37 Shena Z et al. Improved drug targeting of cancer cells by utilizing actively targetable folic acid-conjugated albumin nanospheres. J Pharmacol Res 2011; 63: 51-8.
38. Chacon M et al. Stability and freeze-drying of cyclosporine loaded poly (D, L lactide-glycolide) carriers. Eur J Pharm Sci 1999; 8: 99-07.
39. Nigam P et al. Graphene quantum dots conjugated albumin nanoparticles for targeted drug delivery and imaging of pancreatic cancer. J Mat Chem B 2014; 2: 3190-95.