Carbohydrate Polymers 91 (2013) 48–57

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Development and evaluation of 5-fluorouracil loaded chitin nanogels for treatment of skin cancer

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ARTICLE INFO

Article history:
Received 14 June 2012
Received in revised form 23 July 2012
Accepted 24 July 2012
Available online 9 August 2012

Key words:
Nanogels
Transdermal drug delivery
Chitin
5-Fluorouracil
Skin cancer
Melanoma

ABSTRACT

This study focuses on development and evaluation of 5-fluorouracil (5-FU) loaded chitin nanogels (FCNGs). It formed good, stable aqueous dispersion with spherical particles in 120–140 nm size range and showed pH responsive swelling and drug release. The FCNGs showed toxicity on melanoma (A375) in a concentration range of 0.4–2.0 mg/mL, but less toxicity toward human dermal fibroblast (HDF) cells by MTT assay. Confocal analysis revealed uptake of FCNGs by both cells. From skin permeation experiments, FCNGs showed almost same steady state flux as that of control 5-FU but the retention in the deeper layers of skin was found to be 4–5 times more from FCNGs. Histopathological evaluation revealed loosening of the horny layer of epidermis by interaction of cationically charged chitin, with no observed signs of inflammation and so FCNGs can be a good option for treatment of skin cancers.

1. Introduction

Melanoma, a malignancy of melanocytes mainly found in the skin, is a potentially fatal cancer. It is derived from abnormally proliferating melanocytes, although the process called melanomagenesis has not yet been fully understood (Azarjana, Pjanova, & Čēma, 2008; Zheng et al., 2009). Cutaneous melanoma accounts only for 3% of all skin tumors; however this malignant cancer presents high mortality rates, accounting for 75% of all deaths due to cutaneous malignant neoplasms (Ferrari, Muller, Ribeiro, Maia, & Sanches, 2008; Schwartz et al., 2002; Weinstock, 2006). Compared to the other two common types of skin cancers namely basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), melanoma is more aggressive. Risk factors of melanoma include family history, a previous melanoma incidence, gene polymorphisms, multiple moles, sun sensitivity, immune suppression, alcohol consumption, and exposure to ultraviolet radiation (UV). UV from sunlight induces DNA damage or suppresses the immune system of the skin, thus resulting in skin disorders, including melanoma (Allal & Honnavara, 2001). There are many reports showing the correlation between the increasing number of melanoma in situ (MIS) and UV exposure.

Careful attention toward the pigmented lesions even 1–2 mm, on sun exposed areas facilitate earlier diagnosis which can be treated by less invasive therapy ultimately preventing further complications like metastases. But usually negligence or lack of concern toward such lesions is very common among people thus preventing the chances for early diagnosis. Epidemiological studies have demonstrated heavy alcohol drinking associated with increased risk of melanoma (Boffetta, Nordenvall, Nyrén, & Ye, 2009). The treatment for melanoma mainly involves surgery, chemotherapy and radiation therapy. A combination of surgery with chemotherapy/radiation is usually preferred. Even though it is one among the most preventable and treatable cancer, the prognosis is poor due to low response to conventional chemotherapy. Topical therapy is used in case of MIS as well as for BCC and SCC. This topical therapy, if improved using novel formulations can be a good option for the better management of these cancers as well as other skin diseases. This is because skin is always considered as an important portal of entry for chemicals into the body.

Various colloidal carriers including nanoparticles and lipid vesicles have been studied for improving the notoriously low drug absorption from the skin surface. Many researchers have found that the drug permeation was enhanced by gradual release from the nanoparticles and only a few nanoparticles were able to permeate to the skin passively through the hair follicles while most of

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http://dx.doi.org/10.1016/j.carbpol.2012.07.060

them were primarily restricted to the uppermost stratum corneum layer (Alvarez-Roman, Naik, Kalia, Guy, & Fessi, 2004; Lademann et al., 2007). So the significant penetration barrier provided by the stratum corneum (SC) has to be considered seriously in developing efficient targeted drug delivery systems to the physiological sites in the skin (Shah, Desai, Patel, & Singh, 2012). Nanoformulations with appropriate size and surface charge can have Enhanced Permeation and Retention (EPR) effect through the different layers of skin leading to passive targeting. The nano-sized network of chemically or physically cross linked polymer particles known as the nanogels have many advantages in drug delivery applications. They can aid in creating a uniform dispersion of the nanocarriers in the matrix and increase the contact time which result in enhanced skin penetration of the drug payload (Batheja, Sheihet, Kohn, Singer, & Michniak-Kohn, 2011). Intelligent nanogels that exhibit drastic response to various stimuli have been hot topics in the rapidly growing fields of smart materials and nanomedicine (Hendrickson, Smith, South, & Lyon, 2010). Among various intelligent nanogels, the most extensively studied are those responsive to changes in pH (Du, Sun, Song, Wu, & Wang, 2010; Kim, Kabanov, & Bronich, 2009), temperature (Berndt, Pedersen, & Richtering, 2005), reduction potential (Ryu, Jiwpanich, Chacko, Bickerton, & Thayumanavan, 2010) or their combination (Nobuyunki, Xing-Ping, Françoise Winnik, & Kazunari, 2008; Zhang, Jiang, Zhang, Li, & Liu, 2007).

Chitin based nanogel was first reported by our group, and was prepared by a simple regeneration method without using any organic solvents. It possesses nanosize, cationic charge and pH responsive swelling and drug release at acidic pH. It was proven to have blood compatibility as well as cytocompatibility on a number of normal cell lines (Sabitha et al., 2012; Sanoj Rejinold et al., 2012). It can be a good option for site specific delivery of selected drug for treatment of skin cancers because of the above said properties. 5-Fluorouracil, a pyrimidine analogue, displays a broad spectrum of activity against several solid tumors by interfering with thymidylate synthase (Paul, David, & Siobhaan, 2000). It is one of the oldest antitumor drugs, commonly used in clinical oncology practice. It is widely used in clinical treatment of several solid cancers such as gastrointestinal, pancreas, breast, colorectal, liver and brain cancer (Saif, Syrigos, & Katirtzoglou, 2009). Its topical application in the form of cream and solution is recommended for various superficial skin conditions like multiple actinic keratoses, psoriasis, etc. These formulations are also approved by US FDA for the basal cell carcinoma (BCC). But in these cases the efficacy is limited due to inadequate penetration through SC because of the hydrophilic nature of 5-FU. Attempts have been made to increase the penetration by use of iontophoresis and penetration enhancers (Singh and Jayaswal, 2008; Singh, Singh, & Singh, 2005). But the use of a nanogel formulation is not being reported so far. So the main objectives of this study include preparation and characterization of 5-FU loaded chitin nanogel, evaluation of this for its activity on melanoma cells (A375) by in vitro methods as well the skin permeation studies to measure the penetration and retention effects.

2. Materials and methods

2.1. Materials

Chitin (degree of acetylation 72.4%, molecular weight 150 kDa) was purchased from Koyo chemical Co., Ltd, Japan. Calcium chloride and methanol were purchased from Qualigens, India. 2,4-Dihydroxy-5-fluorouracil 99% was purchased from Sigma Aldrich. Human dermal fibroblast (HDF) cells were obtained from Promocell, Germany and human melanoma cell lines (A375) were received from the National Center for Cell Sciences, Pune, India. The chemicals were used without further purification.

2.2. Preparation of control chitin nanogels

Chitin solution was prepared according to reported method (Tamura, Nagahama, & Tokura, 2006). The chitin nanogels were prepared by regeneration as reported by our group (Sanoj Rejinold et al., 2012).

2.3. Preparation of 5-FU loaded chitin nanogels (FCNGs)

For the preparation of 5-FU loaded chitin nanogels, 5.2 mg of 5-FU was added directly to 5 mL (2.5 mg/mL) CNGs, kept stirring for 5 h to allow for the proper loading of 5-FU into the nanogels. The resulting mixture was then centrifuged to remove the excess drug and was resuspended in an equal volume of water.

2.4. Tagging fluorescent Rhodamine-123 dye with FCNGs (Rhod-FCNGs)

This was done by the addition of 40 μL solution of 1 mg/mL concentration of Rhodamine-123 to 5 mL of FCNGs dispersion under magnetic stirring. This was then probe sonicated for 5 min and continued magnetic stirring for another 2 h and further centrifuged for 30 min at 20,000 rpm to remove the unbound Rhodamine-123.

2.5. 5-FU loading efficiency (LE) of chitin nanogels

The LE of 5-Fu in CNGs was calculated after determining the concentration of unentrapped drug. The supernatant collected after the centrifugation step was analyzed by an HPLC assay (Alsarra & Alarifi, 2004) to determine the concentration of unentrapped drug. The concentration of 5-FU in the sample was calculated against known standards via the method of area under the absorption time curves. The LE was calculated using the formula given below

$$\text{LE}\ \ (\%) = \frac{\text{Weight of drug in nanogel}}{\text{Weight of drug taken initially}} \times 100$$

2.6. Characterizations

The characterizations of all of the samples including control chitin, chitin nanogel, FCNGs and control 5-FU as well were done. FTIR spectral analysis was done using Perkin Elmer Spectrum RX1 Fourier transform infrared spectrophotometer by KBr tablets (1%, w/w of product in KBr) with a resolution of 4 cm^{-1} and 100 scans per sample. Thermal studies were done using S II TG/DTA 6200 EXSTAR. The mean size and size distribution as well as zeta potential of the prepared nanogels (chitin nanogels and FCNGs) were determined by dynamic light scattering (DLS-ZP/Particle Sizer NicompTM 380 ZLS) measurements. Size of the particles was further confirmed using SEM (JEOLJSM-6490LA).

2.7. Swelling studies

The degree of swelling was calculated by finding out weight of swollen nanogels (Li, Wang, Yang, & Li, 2011). The swelling behavior of the control chitin nanogels as well as FCNGs was studied at three different pH conditions (pH 4, 7 and 9 respectively). The swelling ratio was calculated using the following formula after determining the dry as well as wet weight of the lyophilized, pelletized nanogel after sufficient exposure to the corresponding pH solution. The swelling at each pH was studied in triplicate.

$$\text{Swelling ratio} = \frac{W_w - W_o}{W_o}$$

2.8. Drug release studies of FCNGs

The in vitro drug release study was done at two pH values, physiological and acidic since the pH of the skin as well as that at the tumor site is in the acidic range (4–5), moreover the chitin nanogel was shown to have higher swelling at acidic pH (Sabitha et al., 2012). The release study was carried out by dispersing the pellet in 10 mL PBS of 2 different pH values, 7.4 and 4.5 with temperature kept at 37 °C and placed in an incubator shaker at 50 rpm. At predetermined intervals, aliquots of 500 μL were withdrawn and replaced with an equal volume of fresh buffer. These samples were added with 500 μL methanol, mixed well and centrifuged at 10,000 rpm for 15 min to separate the polymeric nanoparticles. The supernatant was filtered using 22 μL syringe filter and 20 μL of the solution was injected into the HPLC system. The isocratic elution with a mobile phase of methanol:water (10:90, v/v) with the pH adjusted to 3.2 using perchloric acid in a C18 column (5 μm, 4.6 mm × 250 mm) was done. At a flow rate of 1 mL/min, the retention time of 5-FU was 1 ± 0.2 min. The detector was set at 260 nm. The concentration of 5-FU in the samples was calculated against known standards via the method of area under the absorption time curves.

2.9. Blood compatibility studies

2.9.1. Hemolysis assay

The hemolysis assay was carried out for control chitin nanogels as well as FCNGs using fresh human blood. 100 μL of sample of concentration ranging from 0.1 to 1 mg/mL were added to 1 mL of blood sample and incubated for 2 h with shaking in an incubator chamber at 37 °C. The rest of the procedure was conducted as per reported literature (Sabitha et al., 2012) and plasma hemoglobin calculated using the following equation.

$$\text{Plasma Hb} = \{(2A_{415}) - [A_{380} + A_{450}] \times 76.25\}$$

The values obtained for samples were compared with that of normal saline as negative control and triton as positive control.

2.9.2. Coagulation assay via PT and APTT measurements

The plasma coagulating effect of control chitin nanogels as well as FCNGs was determined by plasma coagulation assay via two tests, i.e., prothrombin time (PT) and activated partial thromboplastin time (APTT) (Anitha, Chennazhi, Nair, & Jayakumar, 2012). Fresh blood was collected in to ACD containing tubes, centrifuged at 4000 rpm at 25 °C for 15 min to obtain platelet-poor plasma (PPP). The highest concentration of the samples usually used for other tests (1 mg/mL in case of control chitin nanogels and 5 mg/mL of FCNGs) were prepared by dilution with normal saline. 900 μL of PPP was treated with 0.09 μL of sample and kept at 37 °C for 20 min. After the incubation period, the prothrombin time (PT) and activated partial thromboplastin time (APTT) were determined using a coagulation analyzer and reagent kits (CK Prest and Fibriprest from Diagnostica Stago, France).

2.10. Cell culture

Human dermal fibroblast (HDF) cells (Promocell, Germany) were maintained in Minimum Essential Medium (MEM) and A375 (Human melanoma cell lines, NCCS, Pune) were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were incubated in CO_2 incubator with 5% CO_2. After reaching confluency, the cells were detached from the flask with trypsin–EDTA. The cell suspension was centrifuged at 3000 rpm for 3 min and then re-suspended in the growth medium for further studies.

2.10.1. Cell uptake studies of Rhod-FCNGs by confocal microscopy

Confocal microscopy was used to study the uptake of FCNGs by the cells. Rhod-FCNGs were used (Rhodamine-123 excitation 511 nm and emission 534 nm) for this. Cells were seeded at a density of 2×10^4cells per cover slip on acid etched cover slips kept in 24 well plates and incubated for 24 h for the cells to attach on to the cover slips. After 24 h of incubation, media was removed and the wells were carefully washed with PBS. Then Rhod-FCNGs at a concentration of 1 mg/mL was added along with media to triplicate wells and incubated for 24 h. After 24 h of incubation, cover slips were taken out, processed for imaging, there after air dried and mounted on to glass slides with DPX. The slides were then viewed under the confocal microscope (Leica SP 5 II) to study the internalization of FCNGs by the cells.

2.10.2. Cellular localization of Rhod-FCNGs by DAPI/Actin staining-fluorescent microscopy

The A375 and HDF cells were grown on cover slips in 24 well plates with a seeding density of 2×10^4/well for 24 h. The Rhod-FCNGs were treated with cells with a concentration of 1 mg/mL and processed as per reported method (Sanoj Rejinold et al., 2012).

2.10.3. Cytotoxicity studies of FCNGs

Cytotoxicity experiments were carried out on HDF and A375 cell lines by MTT assay (Sabitha et al., 2012). Triplicate samples were analyzed for each experiment.

2.10.4. Apoptosis assay by Flowcytometry

Apoptosis or programmed cell death is a normal component of the development and health of multicellular organisms. Majority of anticancer dugs act by inducing apoptosis on cancer cells by any/many of the signaling pathways. The apoptosis assay was performed by FACS analysis in order to confirm the potential anticancer effect of the formulations.

2.10.5. Apoptosis assay for FCNGs

Annexin V-FITC/PI staining: Phosphatidylserine (PS) translocation from the inner to the outer layer of plasma membrane is one of the important earliest apoptotic features and this PS exposure is detected using an Annexin V-FITC/PI Vibrant apoptosis assay kit (Molecular probes, Eugene, OR) by flow cytometry. Here it was studied for A375 as well as HDF cells as reported by us (Sabitha et al., 2012). Samples were analyzed in triplicate for each experiment.

2.11. Ex vivo skin permeation studies

In case of skin models used for skin permeation studies, porcine skin is highly preferred because of its closest match to human skin comparing the results of several species (Sabitha et al., 2012). The permeation experiment was carried out using full thickness porcine skin in a vertical Franz diffusion (FD) cell.

2.11.1. Preparation of skin samples

Fresh pig ear was collected from a local slaughter house. After thorough washing, the hair on the skin was removed using a razor and the skin was separated from the underlying cartilage and subcutaneous fat using a scalpel and cut into appropriate sizes, wrapped in aluminum foil and stored at −20 °C. The skin samples were stored for a maximum of 1 month only.

2.11.2. Permeation experiment for FCNGs

1 mL of the sample under test (the FCNGs dispersion as well as control 5-FU solution, both containing 500 μg of 5-FU) was added in the donor compartment of the FD cell on the stratum corneum side of the skin and phosphate buffer pH 4.5 was taken in the acceptor compartment. The temperature was kept at 32 °C by a circulating

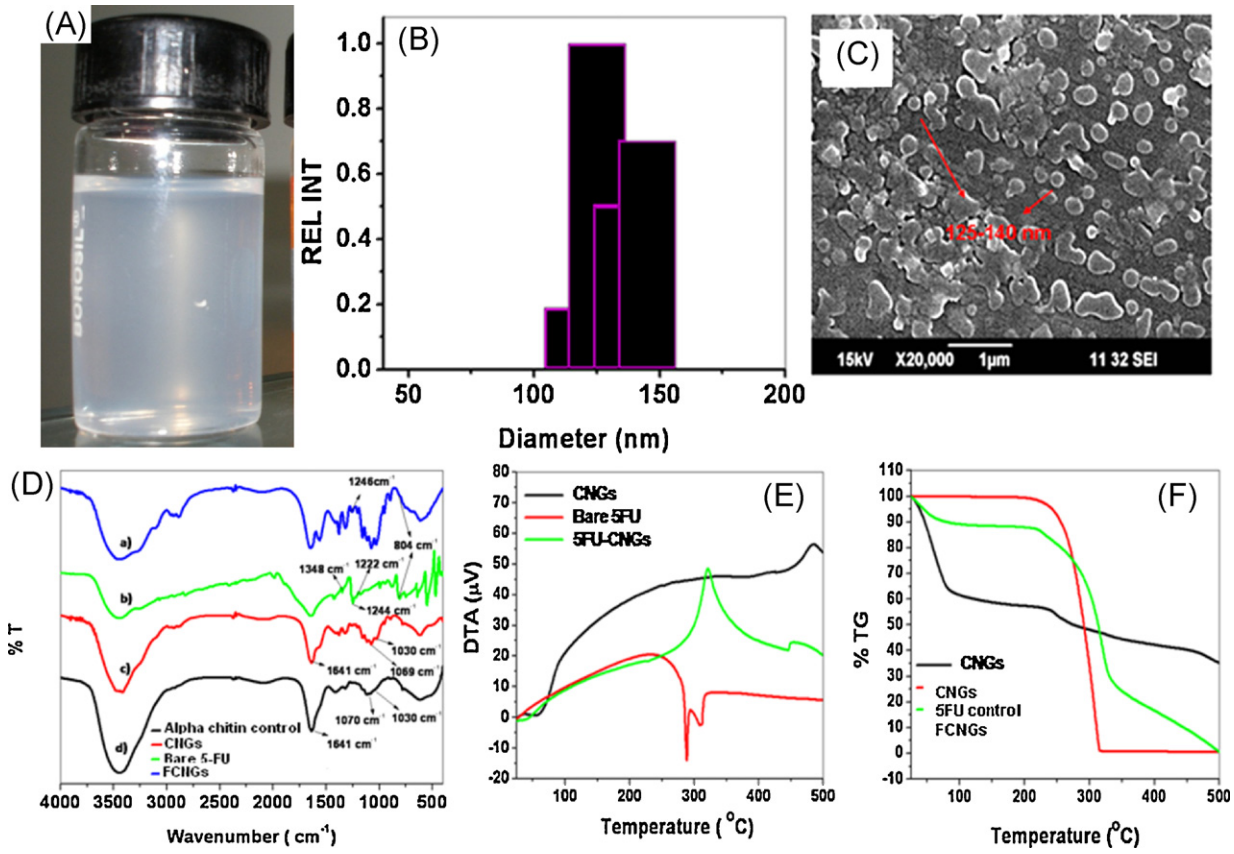

Fig. 1. (A) The stable FCNGs at pH 5.0; (B and C) the size distribution of FCNGs in the DLS and SEM; (D) FTIR spectra and (E and F) DTA and TG analysis of the synthesized materials.

water jacket and acceptor fluid gently stirred with a magnetic stirrer. 0.5mLof the acceptor fluid was taken at 1 h intervals from 0 to 5 h and then at 24 h and replaced with an equal quantity of fresh buffer. The amount of 5-FU penetrated was quantified by HPLC analysis.

2.11.3. HPLC procedure

The amount of 5-FU penetrated through the skin into the acceptor fluid was quantified by an HPLC method (Alsarra & Alarifi, 2004) after mixing the sample collected with equal volume of methanol, centrifugation at 5000 rpm for 30 min and 20 μL of filtered supernatant was injected into the HPLC system under the conditions mentioned earlier to determine the 5-FU concentration.

2.11.4. Drug concentration-depth profiles

The skin samples from the above experiments in triplicate were collected after the exposure period and samples of 1 cm^2 area were cut and placed on a cork disc and were sectioned using a cryotome into 50 mm sections (Karpanen et al., 2008). The cryosections were grouped into three groups as upper, middle and lower sections in three different containers and 5-FU was extracted by incubation with methanol. These were then centrifuged, filtered and HPLC analysis for 5-FU was then carried out as mentioned above.

2.11.5. Histological evaluation

Porcine skin samples were treated with PBS (control), control 5-FU solution, control chitin nanogels and FCNGs respectively in FD cell exactly as in permeation experiments for an exposure period of 10 h. Then, the skin samples were washed properly with PBS and fixed in 10% formaldehyde in PBS. Samples were then processed and vertical sections were taken using a microtome and stained with hematoxylin and eosin (H&E).

2.11.6. Permeation experiment for Rhod-FCNGs

The permeation experiments were repeated with Rhod-FCNGs as well as Rhod-5FU in order to find its localization at different depths of skin by fluorescent imaging. The skin samples from the permeation experiments were collected after the exposure period.

2.11.7. Fluorescent imaging

The skin samples after proper washing were cryosectioned and one section from each control group as well as sample-treated ones were repeatedly treated with xylene to remove water and mounted on to glass slides for fluorescent imaging using DPX. The slides were then viewed under the fluorescent microscope (Olympus-BX-51) to understand the localization of permeated Rhod-FCNGs at different depth of dermal penetration.

3. Results and discussion

3.1. Preparation of 5-fluorouracil (5-FU) loaded chitin nanogels (FCNGs)

The hydrophilic 5-FU was loaded to the prepared CNGs by subjecting to prolonged stirring. This method was found to be better for 5-FU rather encapsulating at the stage of regeneration of chitin as nanogel. 5-FU being hydrophilic in nature and physically adsorbed on the surface of chitin nanogel, a good amount may be lost during repeated centrifugation and water washings done for the preparation of chitin nanogels in order to remove the excess $CaCl_2$ and methanol. This is why it is better to encapsulate 5-Fu to the final

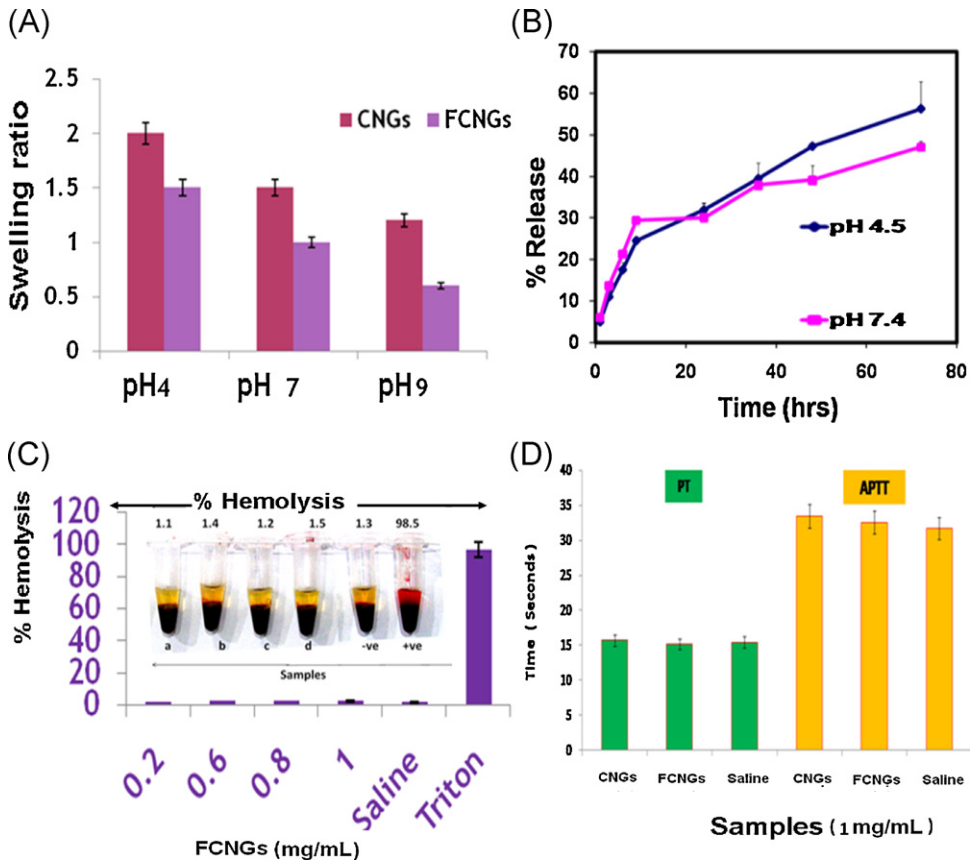

Fig. 2. (A) Swelling ratios of FCNGs in comparison to control CNGs and (B) 5-FU release from the FCNGs at different pH; (C) in vitro hemolysis assay and (D) PT-APTT assay results of FCNGs and control CNGs.

chitin nanogels. The stable aqueous dispersion of FCNGs is shown in Fig. 1A.

3.2. 5-FU loading efficiency of chitin nanogels

The formulation was found to have a loading efficiency of ~90% compared to the reported, maximum loading efficiency of 30% for 5-FU in similar nano and microformulations (Arica, Alis, Kas, Sargon, & Hincal, 2002; Dolores Blanco et al., 2011). The amount of drug with respect to the concentration of the CNGs and incubation time had significant effect on the loading efficiency. It was found that higher amount of drug and longer incubation time resulted in higher loading efficiency. Based on the calculated loading efficiency, the total drug content in the final formulation could be 4.68 mg/12.5 mg chitin.

3.3. Characterization of the control and drug loaded nanogels

The size distribution of the chitin nanogels and FCNGs were studied using DLS and SEM and are described in Fig. 1B and C respectively. The size ranges of the nanogels and their drug loaded counterparts were found to be 50–60 nm and 125–140 nm respectively. As the drug molecules were physically adsorbed on the surface of the nanogel as well as entrapped within the carrier, the size was found to be a bit larger than the control CNGs. The SEM analysis confirmed this size as well as the spherical shape of these nanogels. The zeta potential values for control chitin nanogels and FCNGs were found to be +44.25 and +31.9 mV respectively. On comparing the FTIR spectra of CNGs with FCNGs in Fig. 1D, a peak broadening was seen at 3500 cm^{-1} because of the potential interaction between the nanogel particles. On comparing the 5-FU with FCNGs the peaks at 1222 and 804 cm^{-1} were found to be diminished in intensity because of the amorphous nature of the 5-FU entrapped within the CNGs (Sorina Alexandra, Adi, Corina, & Ronescu, 2011). The intermolecular hydrogen bonding between the carbonyl group and F moiety of the 5-FU with the O-H and free amine groups of the CNGs were observed as shift from 1244 cm^{-1} to 1246 cm^{-1} (Anitha et al., 2011; Sanoj Rejinold et al., 2011). In thermal analysis, 5FU showed single step decomposition and was relatively stable till 200 °C and degraded thermally around 283 °C. The major weight loss occurred between 250 and 320 °C. The FCNGs (Fig. 1F) also started degrading at around the same temperature as 5FU. The loaded nanogel was thermally more stable than the chitin nanogel, which could be because of the association with the drug. The characteristic endotherms of 5-FU were absent in the FCNGs, confirming the amorphous nature of 5-FU within the FCNGs.

3.4. Swelling studies

The swelling ratio was found to be greater at acidic pH since the pK_a of chitin is 6.1 and polyelectrolyte gels usually swell at pH below its pK_a. This was found to be same even after the drug loading. But the swelling was found to be lesser for FCNGs compared to the control nanogels (Fig. 2A) since the drug conjugation reduces the free reactive functional groups.

3.5. In vitro drug release studies

The drug release was found to be higher at acidic pH for FCNGs as shown in Fig. 2B. In 9 h, about 25% of the drug was released at acidic pH and about 30% at neutral pH. At about 72 h, 55% of the drug was released at acidic pH and at neutral pH it was found to

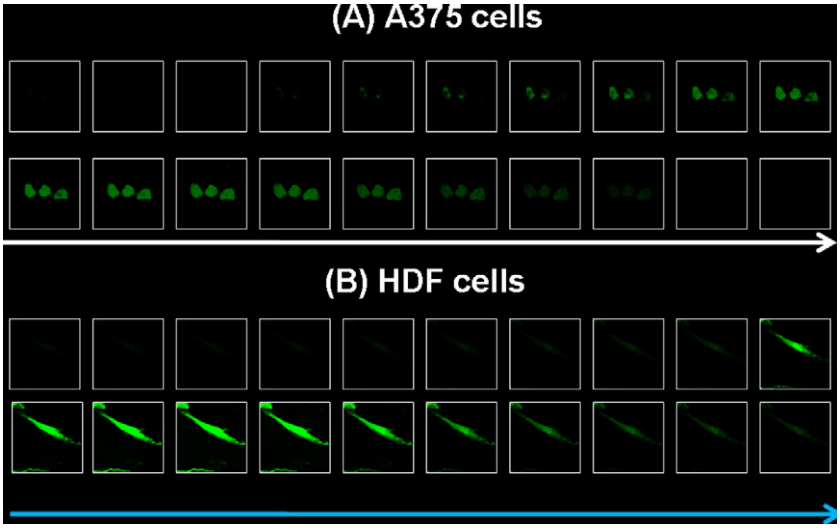

Fig. 3. Cellular localization of FCNGs on A375 and HDF cells respectively after 6 h incubation period by confocal microscopy.

be around 40%. The possible mechanism of drug release from chitin nanogels has been already reported in our previous work (Sabitha et al., 2012).

3.6. Blood compatibility studies of FCNGs

The FCNGs were found to be safe in terms of hemolytic ratio (Fig. 2C) which was very low as compared to the critical safe hemolytic ratio of biomaterials according to ISO/TR 7406. The prothrombin as well as the activated partial thrombin time for both the control chitin nanogels as well as the FCNGs was found to be within the normal limits and comparable with that of the negative control as clearly indicated in Fig. 2D. These results indicate that there will be no risk of hemolysis or coagulation even if systemic delivery is achieved through the transdermal route. As there was no hemolysis observed even at the higher concentration, the possibility for positively charged nanogels to interact with sialic acid residues on RBC seems to be negligible (Yallapu, Ebeling, Chauhan, Jaggi, & Chauhan, 2011). This could be the reason why no clumping or RBC rupture was observed.

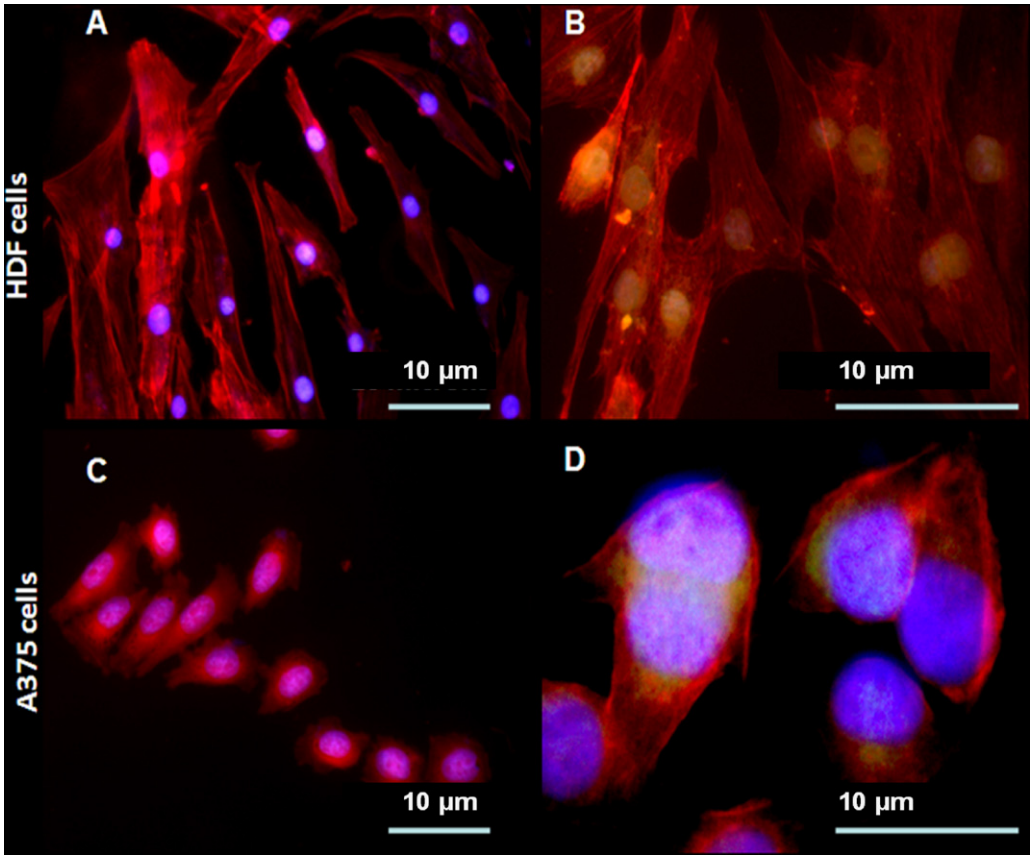

Fig. 4. Fluorescent images showing internalization of Rhod-FCNGS by DAPI/Actin staining.

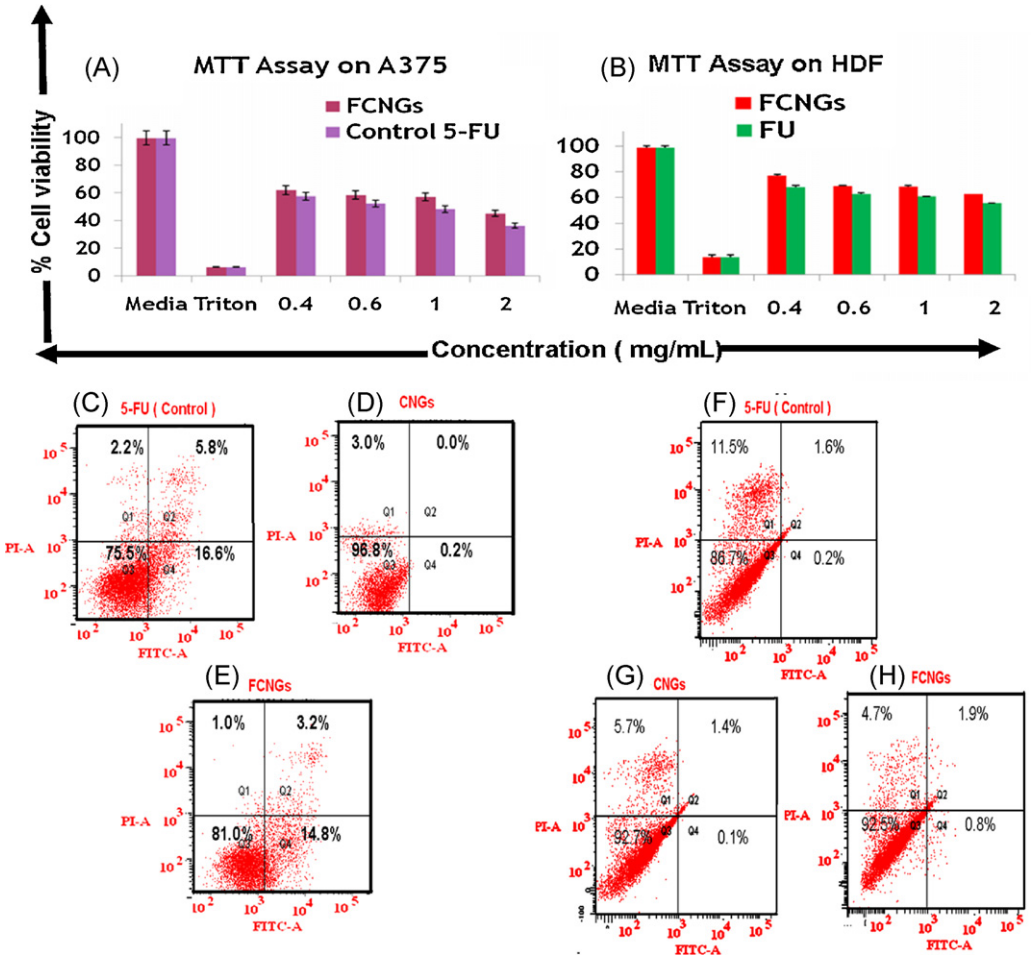

Fig. 5. (A and B) The results of MTT assay on A 375 and human dermal fibroblast cells. (C–E) The apoptotic profile of 5-FU; CNGs and FCNGs on A375 and (F–H) the apoptotic profile of 5-FU; CNGs and FCNGs on HDF cells.

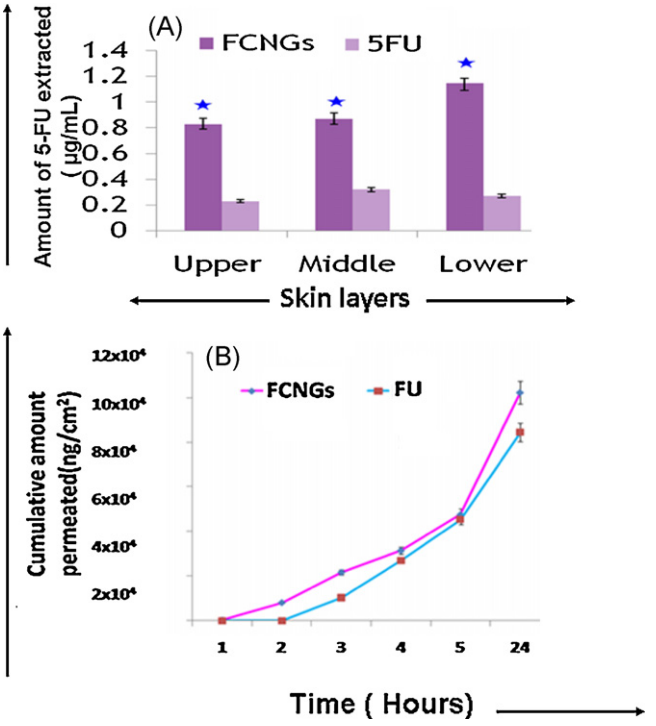

Fig. 6. (A) Penetration experiments showing concentration-depth profile after 24 h exposure (values reported are mean ± S.D., $n = 3$); (B) plot of cumulative amount of 5-FU permeated vs time for control 5-FU solution and FCNGs.

3.7. Cell uptake studies

The confocal images in Fig. 3 clearly confirm that Rhod-FCNGs are taken up by both A375 as well as HDF cells. Internalization of Rhod-FCNGs was studied by DAPI/Actin staining using fluorescent microscopy which again shows the internalization of these particles between the nucleus and exoskeleton of both the cells (Fig. 4). As discussed earlier, 5-FU act by inhibiting the enzyme thymidylate synthase, the primary role of which is production of deoxythymidine triphosphate for DNA synthesis, a process largely considered to occur in the cytoplasm (Gustavson, Molinaro, Tedeschi, Camp, & Rimm, 2008). So the internalization observed within the cytoplasm very well supports the mechanism of action of 5-FU. There are many reports suggesting for a receptor mediated uptake of chitin derivatives in plant and mammalian cells, but the mechanism is unclear (Feng, Zhao, & Yu, 2004). This is an added advantage for these materials for use in cancer drug delivery.

3.8. Cytotoxicity studies

The MTT assay results indicate that FCNGs at the highest concentration selected showed about 50% cell death in case of A375 and mild toxicity was seen toward human dermal fibroblast cells as shown in Fig. 5A and B, respectively. 5-FU is a purely S-phase active drug with no activity when cells are in G0 or G1 phase. 5-FU has a complicated mechanism of action with several enzymes involved in its metabolic activation. It inhibits thymidylate synthase as its

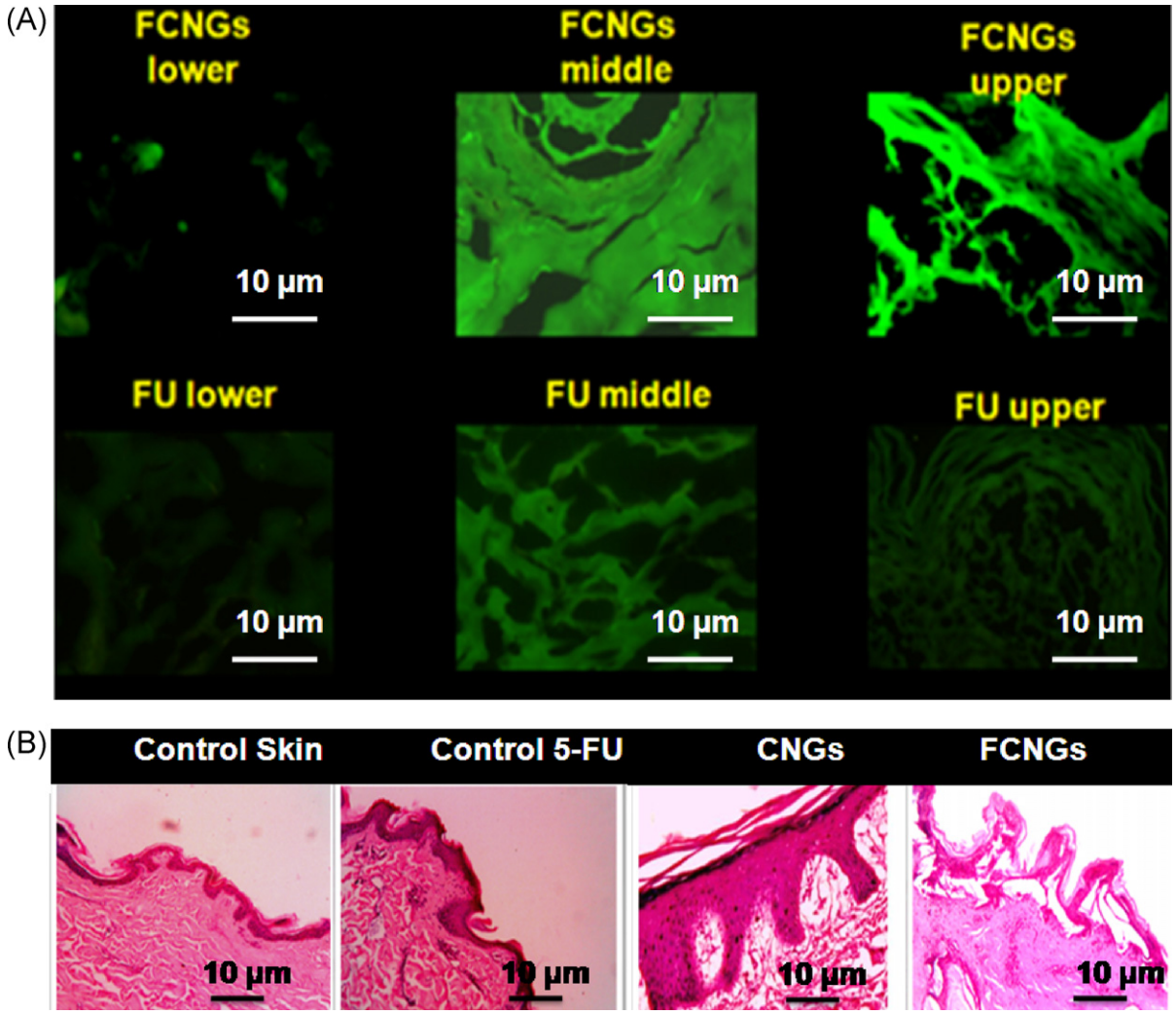

Fig. 7. (A) Skin localization of Rhod-FCNGs as well as control 5-FU by fluorescent microscopy after 6 h exposure; (B) images showing results of histopathology studies for control 5-FU solution, control chitin nanogels and FCNGs.

main mechanism of action leading to depletion of dTTP. It also cause DNA damage (both single and double strand breaks) during S phase due to misincorporation FdUTP into DNA and this DNA damage can occur in all the phases of cell cycle in proliferating cells and in turn affects proliferation and survival (De Angelis, Svendsrud, Kravik, & Stokke, 2006). 5-FU is a drug of choice for treatment of BCC and SCC in the form of a topical 5% cream and also there are reports about its efficacy as an intralesional agent offering deeper penetration and better management of these tumors (Laurie, Misha, & Whitney, 2011).

3.9. Apoptosis assay by flow cytometry

The apoptotic profile of FCNGs and control 5-FU on A375 and HDF cells are shown in Fig. 5C–H. As indicated in Fig. 5C and E, 5-FU and FCNGs showed only a mild cytotoxic effect on A 375 cells. 5-FU induces apoptosis by influencing the caspase cascade as well as p 53 expression. Even though it is used topically for BCC and SCC, its efficacy against melanoma is never reported earlier. It is again a well known fact that many cells develop resistance to treatment with 5-FU. Overexpression of thymidylate synthase has been shown to be associated with 5-FU resistance but alterations in other factors like crucial genes in cell cycle and apoptotic regulatory pathway also contribute to this. Studies show that incorporation of 5-FU along with other drugs increase caspase dependent apoptosis in certain cells (De Angelis et al., 2006; Zoli et al., 2005).

The apoptotic results on HDF cells are shown in Fig. 5F–H. From this it is clear that the toxicity of 5-FU on HDF is lowered after encapsulation in the chitin nanogels. The reduced toxicity of FCNGs may be due to the antioxidant potential of chitin since such reduction in toxicity on HDF cells with co administration of antioxidants is well established for the phyto drug curcumin (Sabitha et al., 2012).

3.10. Skin permeation studies

The skin penetration of 5-FU from FCNGs was found to be almost same as that from control 5-FU solution. 5-FU is a hydrophilic drug, it may penetrate through the aqueous pores and that may be the reason for lack of further enhancement in penetration. The enhancement of skin penetration of hydrophilic drugs has always been a big challenge and in this case also the enhancement could be brought about only in retention not in the permeation through the full thickness skin. The flux determined from Fig. 6B was found to be same for FCNGs compared to the control 5-FU solution (Table 1), which means the enhancement ratio (Er) for FCNGs was 1. The chitin nanogels can interact with the anionic lipids and keratin, but in case of FCNGs the hydrophobicity of chitin is reduced or masked due to the surface adsorption of the hydrophilic 5-FU thereby

Table 1
Permeability data (mean ± S.D., $n = 3$) calculated for control 5-FU solution and FCNGs (concentration taken is 1×10^6 ng/mL).

Samples	Steady state flux, J ($ng\,cm^{-2}\,h^{-1}$)	Permeability constant, P ($\times 10^{-3}\,cm\,h^{-1}$)
Control 5-FU	$2.8 \times 10^4 \pm 0.25$	28 ± 0.25
FCNGs	$3.0 \times 10^4 \pm 0.45$	30 ± 0.45

reducing the above said interactions. That may be another reason why the enhancement in penetration was poor in this case.

It is very much clear from the fluorescent images (Fig. 7A) as well as the concentration depth profile shown in Fig. 6A that the retention of 5-FU at the deeper layers of the epidermis and dermis is 4–5 times greater in the case of FCNGs as compared to the control 5-FU solution (statistically significant when analyzed by Student-*T* test). Irrespective of the poor results in permeation, the enhanced retention is observed due to the cationic charge of the FCNGs. Chitosan, a mucoadhesive and positively charged polysaccharide is reported to act as an enhancer to increase skin permeability of formulations (Smith, Wood, & Dornish, 2004). The suggested mechanism is by a more loosened accumulation of keratin in the stratum corneum. Chitin is also very much in use in cosmetic products for skin application. This means that chitin also share these properties of chitosan and the solubility concerns of chitin which limits its applications for drug delivery is being taken care in this nanogels preparation which forms a very good stable aqueous dispersion. This retention at deeper layers of skin is very much advantageous as melanocytes are located at the deeper layers of epidermis.

3.11. Histological evaluation

The images of the histopathological evaluations as seen in Fig. 7B shows the loosening of the lipid bilayer in the epidermis as well as ablation of the stratum corneum barrier is clear in case of skin treated with chitin nanogels as well as FCNGs whereas such a change is not there in case of skin treated with control 5-FU solution. Any carrier used for transdermal or percutaneous delivery must be capable of crossing the stratum corneum barrier in order to reach the systemic circulation or for localization at the disease sites. Chitin nanogel as explained earlier has proven effective in this and the histopathological evaluation clearly shows the altered organization of SC, at the same time was free from toxicity in terms of erythema or edema. An enhancement in skin penetration and retention is achieved in the study without toxicity and irritation, which is an additional advantage since most of the chemical enhancers used for this purpose result in some toxicity.

4. Conclusions

The hydrophilic drug 5-FU was effectively loaded on the chitin nanogels to prepare FCNGs. The FCNGs were characterized by DLS, SEM, FTIR and TG/DTA which confirmed the nanosized spherical particles with intermolecular hydrogen bonding and the drug loaded nanogels were thermally more stable compared to control chitin nanogels. FCNGs showed increased swelling and drug release at acidic pH possibly due to the protonation of free amine groups. The blood compatibility was confirmed by hemolysis and coagulation assays. Compared to our previous chitin nanogel formulation with curcumin, FCNGs showed reduced cytotoxicity on A375 cells. Similarly the enhancement in penetration through the full thickness of skin was also negligible, but the retention in deeper layers of skin was found to be 4–5 times greater than the control 5-FU solution. As the surface adsorption of 5-FU might have resulted in reducing the possible interactive sites of chitin as well as exhibiting a more hydrophilic nature may be the reason for this. 5-FU is used as topical cream for skin cancers like BCC and SCC and so the FCNGs can be a better option for the treatment of these skin cancers.

Acknowledgments

The authors are thankful for financial support from Department of Science and Technology (DST) through the project '*Cancer Nanotheragnostics*' (SR/NM/NS-99/2009). The great support from all colleagues at Amrita Center for Nanosciences and Molecular Medicine and Amrita School of Pharmacy is acknowledged here.

References

Allal, O., & Honnavara, N. A. (2001). A model for UV-induction of skin cancer. *Journal of Biomedicine and Biotechnology*, *1*, 5–6.
Alsarra, I. A., & Alarifi, M. N. (2004). Validated liquid chromatographic determination of 5-fluorouracil in human plasma. *Journal of Chromatography B*, *804*, 435–439.
Alvarez-Roman, R., Naik, A., Kalia, Y. N., Guy, R. H., & Fessi, H. (2004). Enhancement of topical delivery from biodegradable nanoparticles. *Pharmaceutical Research*, *21*, 1818–1825.
Anitha, A., Deepagan, V. G., Divya Rani, V. V., Deepthy, M., Nair, S. V., & Jayakumar, R. (2011). Preparation, characterization, in vitro drug release and biological studies of curcumin loaded dextran sulphate-chitosan nanoparticles. *Carbohydrate Polymers*, *84*, 1158–1164.
Anitha, A., Chennazhi, K. P., Nair, S. V., & Jayakumar, R. (2012). 5-Flourouracil loaded *N*,*O*-carboxymethyl chitosan nanoparticles as an anticancer nanomedicine for breast cancer. *Journal of Biomedical Nanotechnology*, *8*, 29–42.
Arica, B., Alis, S. C., Kas, H. S., Sargon, M. F., & Hincal, A. (2002). A 5-fluorouracil encapsulated alginate beads for the treatment of breast cancer. *International Journal of Pharmaceutics*, *242*, 267–269.
Azarjana, K., Pjanova, D., & Čēma, I. (2008). Melanoma: The influence of early diagnostics to the prognosis of the disease. *Doctus*, *5*, 5–9.
Batheja, P., Sheihet, L., Kohn, J., Singer, A. J., & Michniak-Kohn, B. (2011). Topical drug delivery by a polymeric nanosphere gel: Formulation optimization and *in vitro* and *in vivo* skin distribution studies. *Journal of Controlled Release*, *149*, 159–167.
Berndt, I., Pedersen, J. S., & Richtering, W. (2005). Structure of multiresponsive "intelligent" core-shell microgels. *Journal of American Chemical Society*, *127*, 9372–9373.
Boffetta, P., Nordenvall, C., Nyrén, O., & Ye, W. (2009). A prospective study of gout and cancer. *European Journal of Cancer Prevention*, *18*, 127–132.
De Angelis, P. M., Svendsrud, D. H., Kravik, K. L., & Stokke, T. (2006). Cellular response to 5-fluorouracil (5-FU) in 5-FU resistant colon cancer cell lines during treatment and recovery. *Molecular cancer*, *5*, 20. http://dx.doi.org/10.1186/1476-4598-5-20
Dolores Blanco, M., Guerrero, S., Benito, M., Fernández, A., Teijón, C., Olmo, R., et al. (2011). *In vitro* and *in vivo* evaluation of a folate-targeted copolymeric submicrohydrogel based on *N*-isopropylacrylamide as 5-fluorouracil delivery system. *Polymers*, *3*, 1107–1125.
Du, J. Z., Sun, T. M., Song, W. J., Wu, J., & Wang, J. (2010). A tumor-acidity-activated charge conversional nanogel as an intelligent vehicle for promoted tumoral-cell uptake and drug delivery. *Angewandte Chemie International Edition*, *49*, 3621–3626.
Feng, J., Zhao, L., & Yu, Q. (2004). Receptor-mediated stimulatory effect of oligochitosan in macrophages. *Biochemical and Biophysical Research Communications*, *317*, 414–420.
Ferrari, N. M. J, Muller, H., Ribeiro, M., Maia, M., & Sanches, J. A. J. (2008). Cutaneous melanoma: Descriptive epidemiological study. *Sao Paulo Medical Journal*, *126*, 41–47.
Gustavson, M. D., Molinaro, A. M., Tedeschi, G., Camp, R. L., & Rimm, D. L. (2008). AQUA analysis of thymidylate synthase reveals localization to be a key prognostic biomarker in 2 large cohorts of colorectal carcinoma. *Archives of Pathology and Laboratory Medicine*, *132*, 1746–1752.
Hendrickson, G. R., Smith, M. H., South, A. B., & Lyon, L. A. (2010). Design of multiresponsive hydrogel particles and assemblies. *Advanced Functional Materials*, *20*, 1697–1712.
Karpanen, T. J., Worthington, T., Conway, B. R., Hilton, A. C., Elliott, T. S. J., & Lambert, P. A. (2008). Penetration of chlorhexidine into human skin. *Antimicrobial Agents and Chemotherapy*, *52*, 3633–3636.
Kim, J. O., Kabanov, A. V., & Bronich, T. K. (2009). Polymer micelles with cross-linked polyanion core for delivery of a cationic drug doxorubicin. *Journal of Controlled Release*, *138*, 197–204.
Lademann, J., Richter, H., Teichmann, A., Otberg, N., Blume-Peytavi, U., Luengo, J., et al. (2007). Nanoparticles-an efficient carrier for drug delivery into the hair follicles. *European Journal of Pharmaceutics and Biopharmaceutics*, *66*, 159–164.
Laurie, M. G., Misha, D. M., & Whitney, A. H. (2011). Intralesional agents in the management of cutaneous malignancy: A review. *Journal of American Academy of Dermatology*, *64*, 413–422.
Li, N., Wang, J., Yang, X., & Li, L. (2011). Novel nanogels as drug delivery systems for poorly soluble anticancer drugs. *Colloids and Surfaces B: Biointerfaces*, *83*, 237–244.

Nobuyunki, M. N., Xing-Ping, Q., Françoise Winnik, M., & Kazunari, A. (2008). Dual stimuli-responsive nanogels by self-assembly of polysaccharides lightly grafted with thiol-terminated poly (N-isopropylacrylamide) chains. *Macromolecules*, *41*, 5985–5987.

Paul, A. M., David, A. W., & Siobhaan, M. K. (2000). Sustained release of 5-fluorouracil from polymeric nanoparticles. *Journal of Pharmaceutics and Pharmacology*, *52*, 1451–1459.

Ryu, J. H., Jiwpanich, S., Chacko, R., Bickerton, S., & Thayumanavan, S. (2010). Surface functionalizable polymer nanogels with facile hydrophobic guest encapsulation capabilities. *Journal of American Chemical Society*, *132*, 8246–8247.

Sabitha, M., Sanoj Rejinold, N., Amrita, N., Vinoth Kumar, L., Shantikumar, V. N., & Jayakumar, R. (2012). Curcumin loaded chitin nanogels for skin cancer treatment via the transdermal route. *Nanoscale*, *4*, 239–250.

Saif, M. W., Syrigos, K. N., & Katirtzoglou, N. A. (2009). A promising new oral fluoropyrimidine derivative. *Expert Opinion on Investigational Drugs*, *18*, 335–348.

Sanoj Rejinold, N., Amrita, N., Sabitha, M., Chennazhi, K. P., Tamura, H., Nair, S. V., et al. (2012). Synthesis, characterization and *in vitro* cytocompatibility studies of chitin nanogels for biomedical applications. *Carbohydrate Polymers*, *93*, 936–942.

Sanoj Rejinold, N., Muthunarayanan, M., Divyarani, V. V., Sreerekha, P. R., Chennazhi, K. P., Nair, S. V., et al. (2011). Curcumin-loaded biocompatible thermo responsive polymeric nanoparticles for cancer drug delivery. *Journal of Colloid and Interface Science*, *360*, 39–51.

Schwartz, J. L., Wang, T. S., Hamilton, T. A., Lowe, L., Sondak, V. K., & Johnson, T. M. (2002). Thin primary cutaneous melanomas. *Cancer*, *95*, 1562–1568.

Shah, P. P., Desai, P. R., Patel, A. R., & Singh, M. S. (2012). Skin permeating nanogel for the cutaneous co-delivery of two anti-inflammatory drugs. *Biomaterials*, *33*, 1607–1617.

Singh, B. N., & Jayaswal, S. B. (2008). Iontophoretic delivery of 5-fluorouracil through excised human stratum corneum. *Drug Discoveries and Therapeutics*, *2*, 128–135.

Singh, B. N., Singh, R. B., & Singh, J. (2005). Effect of ionization and penetration enhancers on the transdermal delivery of 5-fluorouracil through excised human stratum corneum. *Drug Discovery and Therapeutics*, *298*, 98–107.

Smith, J., Wood, E., & Dornish, M. (2004). Effect of chitosan on epithelial cell tight junctions. *Pharmaceutical Research*, *21*, 43–49.

Sorina Alexandra, G., Adi, G., Corina, & Ronescu. (2011). Systems based on dendrimers and antitumoral drug synthesized by non-covalent method. The influence of dendrimers generation. *Materiale Plastice*, *48*, 17–22.

Tamura, H., Nagahama, H., & Tokura, S. (2006). Preparation of hydrogels under mild conditions. *Cellulose*, *13*, 357.

Weinstock, M. A. (2006). Cutaneous melanoma: Public health approach to early detection. *Dermatologic Therapy*, *19*, 26–31.

Yallapu, M. M., Ebeling, M. C., Chauhan, N., Jaggi, M., & Chauhan, S. C. (2011). Interaction of curcumin nanoformulations with human plasma proteins and erythrocytes. *International Journal of Nanomedicine*, *6*, 2779–2790.

Zhang, J. Y., Jiang, X., Zhang, Y. F., Li, Y. T., & Liu, S. Y. (2007). Facile fabrication of reversible core cross-linked micelles possessing thermosensitive swellability. *Macromolecules*, *40*, 9125–9132.

Zheng, D., Li, X., Xu, H., Lu, X., Hu, Y., & Fan, W. (2009). Study on docetaxel-loaded nanoparticles with high antitumor efficacy against malignant melanoma. *Biochimica et Biophysica Acta Sinica*, *41*, 578–587.

Zoli, W., Ulivil, P., Tesei, A., Fabbri, F., Rosetti, M., Maltoni, R., et al. (2005). Addition of 5-fluorouracil to doxorubicin-paclitaxel sequence increases caspase dependent apoptosis in breast cancer cell lines. *Breast Cancer Research*, *7*, R681–R689.