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

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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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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.

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