Published in final edited form as:

Int J Biol Macromol. 2019 May 01; 128: 740–751. doi:10.1016/j.ijbiomac.2019.01.147.

pH triggered and charge attracted nanogel for simultaneous evaluation of penetration and toxicity against skin cancer: *In-vitro* and *ex-vivo* study

Prashant Sahu^a, Sushil K. Kashaw^{a,b,*}, Samaresh Sau^b, Varun Kushwah^c, Sanyog Jain^c, Ram K. Agrawal^a, Arun K. Iyer^{b,d,**}

^aDepartment of Pharmaceutical Sciences, Dr. Harisingh Gour Central University, Sagar, MP, India

^bUse-inspired Biomaterials & Integrated Nano Delivery (U-BiND) Systems Laboratory, Department of Pharmaceutical Sciences, Wayne State University, Detroit, MI, USA

^cCentre for Pharmaceutical Nanotechnology, Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, SAS Nagar (Mohali), Punjab, India

^dMolecular Therapeutics Program, Karmanos Cancer Institute, Detroit, MI, USA

Abstract

The current research is focused to develop and investigate the toxicity and penetration potential of biocompatible chitosan nanogel encapsulating capecitabine by ionic interaction mechanism exhibiting pH triggered transdermal targeting. The nanogel (CPNL) was synthesized by ion gelation mechanism using Pluronic F 127 and surface decoration by Transcutol as non-ionic penetration enhancer. The CPNL possesses fine morphology and nano size range when evaluated by TEM, SEM and DLS analysis with cationic charge and slightly acidic pH assayed by zeta potential and pH analysis. It showed pH responsive drug release characteristics mimicking the skin cancer micro-environment. The MTT assay and apoptotic index of CPNL on HaCaT cell line elaborated optimal cell toxicity and retention on 24 h of exposure. The *ex-vivo* skin penetration analysis exhibited noteworthy diffusion and penetration caliber through concentration depth profile, steady state flux and fluorescent skin imaging on porcine tissue. Overall outcomes suggested CPNL as a potent alternative biocompatible, transdermal nanotherapy against skin cancer displaying significant penetration caliber with enhance toxicity on cancerous cell.

Keywords

Capecitabine; Nanogel; Skin cancer

*Correspondence to: S. Kashaw, Department of Pharmaceutical Sciences, Dr. Harisingh Gour Central University, Sagar, MP 470003, India. sushilkashaw@gmail.com (S.K. Kashaw). **Correspondence to: A. Iyer, Use-inspired Biomaterials & Integrated Nano Delivery (U-BiND) Systems Laboratory, Department of Pharmaceutical Sciences, Wayne State University, Detroit, MI, USA.

Conflict of interest

We declare no conflicts of interest.

1. Introduction

Antimetabolites refers to the class of cytotoxic agents which are similar in structures and exhibits toxic effects on tumor cells, growth and division by inhibiting metabolic pathway of cell [1]. Antimetabolites play a vital role against skin cancer in which 5-Fluorouracil (5-FU) is the most familiar and widely used. In modern medical era, Capecitabine is a novel choice of antimetabolite over 5-FU, displaying close compatibility with 5-FU by converting itself to fluorouracil on metabolism [2]. For combating skin cancer like SCC and BCC, 5-FU was employed with varied topical concentration ranging 1–5% w/v. The key mechanism associated with fluorouracil is the inhibition of thymidylate synthesis, resulting in blockage of DNA production [3]. Skin cancer arises from skin and divided into three main categories, i.e., Squamous cell carcinoma (SCC), Basal cell carcinoma (BCC) and Melanoma [4]. SCC and BCC appear as painless clumps and ulcers with shiny white exteriors revealing small blood vessels. Melanoma spreads slowly in the forms of moles cluster and regards as the most aggressive form of skin cancer. Various tactics and mechanism have been adopted for the effective transdermal chemotherapy (TC) against skin cancer. The TC is based on enhanced penetration and retention (EPR) phenomenon by virtue of passive and active targeting mechanism [5]. The skin barricades occur as the first check for any transdermal drug delivery system. They exhibit as “brick and mortar” type configurations of keratin rich corneocytes in the intracellular matrix. The “diffusion” regards as the basic mechanism for the effective transdermal drug delivery [6]. The hydrophilic molecule diffuses intercellularly in the aqueous region at the intracellular keratin filament. The lipophilic molecule diffuses intercellularly at lipid atmosphere between filaments [7]. Nanogels demonstrate nano-sized version of hydrogel, synthesized by physically or chemically crosslinking phenomenon and can swell in a good medium [8]. Numerous bioactive and cytotoxic agents can be encapsulated in nanogel unveiling competent targeting and controlled drug release [9]. We discovered less toxic, reduced dose transdermal nanotherapy against skin cancer utilizing established antimetabolite and existing synthesis technique with enhanced penetration and cytotoxic efficiency *in-vitro* [10]. The synthesized Chitosan- Capecitabine nanogel (CPNL) on surface modification exhibits pH triggered drug release in the acidic tumor microenvironment. This pH responsive phenomenon of nanogel facilitates sustained release of Capecitabine at targeted sites (tumor site). The amalgam of the biocompatible synthesis technique and surface modification of CPNL displayed acid activation and ion attraction mechanism [11]. The ionic attraction between cationic CPNL and anionic tumor cell membrane facilitate acid degradation of chitosan matrix. This pH triggered nanogel degradation exhibited on-site release of Capecitabine showing cytotoxic effect on tumor cell by EPR mechanism (Fig. 1) [12]. The EPR refers to the passive targeting technique exhibiting leaky tumor vascular organization permeable for blood flow due to disordered endothelial cell layers. This leaky vasculature cell organization enables enhanced uptake of nanoparticles and release of cytotoxic pay-load through endocytosis. This EPR based on-site drug release showed better retention and higher therapeutic benefits of CPNL at the targeted site avoiding local cellular toxicity [13–14]. Therefore, the developed nanotherapy opens novel platform in TDDS exhibiting on-site drug release and maintaining cellular safety margins with enhanced therapeutic potential against skin cancer [15].

2. Material and methods

2.1. Materials

Chitosan of medium molecular weight with a 75% degree of de-acetylation (medical grade) and Glacial Acetic acid (GAA) 100% ultra-pure was procured from Hi-media chemical Ltd. Mumbai, India. Sodium tripolyphosphate (TPP), Transcutol and Pluronic F-127 were purchased from Sigma Aldrich, Bengaluru, India. Capecitabine was obtained as a benevolent gift from Khandelwal labs Ltd., Mumbai, India. Phosphotungestic acid and NaCl were acquired from Rankem Chemicals Ltd., New Delhi India. Deionized water was produced from milli-Q Synthesis (18 MQ, Millipore). All other reagents and chemical were of analytical grade and used as received.

2.2. Synthesis of biocompatible nanogel

Ionotropic gelation technique was employed for the synthesis of CPNL, encapsulating Capecitabine aiding Pluronic F-127 as a copolymer solvent system. The surface decoration of CPNL was performed by adding Transcutol which act as a nonionic penetration enhancer. Accurately weighed 100 mg of Capecitabine and 0.4% w/v of Chitosan were dissolved in 1% v/v aqueous glacial acetic acid (GAA) solution. Drop wise addition of 0.4% Sodium tripolyphosphate solution (TPP) was performed in drug polymer solution at the rate of 2 ml/min (12 ml TPP in 20 ml drug polymer solution). The obtained particles dispersion were sonicated using a probe sonicator (S-4000; Misonix, Farmingdale, NY) at medium amplitude (50%) for 5 min to obtain nano sized particles [16]. The dispersion was then filtered through a 0.2 μ m hydrophilic filter (Minsart, Sartorius) for isolation of smaller nanosize particles in order to achieve maximum transportation at targeted site. The nanoparticles filtration also facilitates unwanted adsorption of drug loaded nanoparticles at non-targeted site, avoiding local cellular and tissue toxicity [17]. The nano sized particles, thus obtained were carefully purified by ultrafiltration (Amicon 8200 with a millipore PBMK membrane, MWCO 300000) against double distilled water at optimal temperature. The ultrafiltration facilitates elimination of residual of unreacted solvent and unbound drug. Pluronic F 127 and Transcutol (22% w/v & 24% v/v) was dissolved in distil water and incubate for 24 h at 30 °C respectively. Dried capecitabine nanoparticles were added in the Pluronic 127 and Transcutol solvent system with continuous stirring by using magnetic stirrer (REMI) for about 2 h at 1000 rpm for the final synthesis of CPNL (Fig. 1) [18].

3. Nanogel characterization

3.1. Particle size, zeta potential, pH and morphology

The developed CPNL particle size and surface charge was evaluated by Malvern Zetasizer 3000 particle size and zeta potential analyzer (Malvern Instruments, Bedfordshire, UK). The Zeta potential of CPNL was investigated by applying the principle of electrophoretic mobility of molecules in an applied electrical field. The concentration of nanogel was adjusted at 0.01% w/v using distilled water or in 0.01 M sodium chloride solution for potential calculation [19]. The pH was measured by using a digital pH meter (HI-TECH WATER TECH. New Delhi, India). The pH meter was first calibrated using buffer tablet, the pH meter was dipped in a beaker containing CPNL on post calibration [20]. The

formulation, evaluation was triplicated and the measurement was repeated thrice with an average value along with SD was reported.

3.2. Dynamic light scattering (DLS)

The CPNL nanoformulation was assayed for the Dynamic Light Scattering (DLS) analyzing mean diameter and PDI using Brook-heaven BI 9000 AT instrument system (Brookhaven Instrument Corporation, USA). The DLS analysis was conceded out for the more distinct and significant evaluation of CPNL. The DLS evaluation was done at wavelength 304 nm at temperature of 25 °C [21].

3.3. Transmission electron microscopy (TEM)

Transmission Electron Microscopy of CPNL was analyzed by using Hitachi H-7500 TEM analyzer. TEM descriptions were obtained to envisage the shape and structure of CPNL. The CPNL was coated with 2.5% w/v of phospho-tungstic acid (PTA) solution and placed in a copper disc grid [22]. The grid was then desiccated in 60 watt LED lamp (Philips, India Ltd.) and was finally positioned into the disc holder and scanned for TEM evaluation.

3.4. Scanning electron microscopy (SEM)

From the structural point of view, morphology and orientation of prepared CPNL nanogel were analyzed by Scanning Electron Microscopy (SEM), Nova Nano SEM 450, Germany. Prior to the SEM evaluation, the CPNL was lyophilized by using freeze dry lyophilizer (REMI, New Delhi, India). The dried nanogel was then placed on a SEM stub by employing double sided adhesive tape at 50 mA 5–10 min *via* sputter (KYKY SBC-12, Beijing, China). A Scanning Electron Microscope aided with secondary electron detector was employed to obtain digital images of the developed nanogel system [23].

3.5. Gelation time and temperature

Gelation temperature of CPNL was analyzed by putting the nanogel in 10 ml vials followed by insertion in a cold water bath at temperature 4 °C. The temperature was then elevated at 2 °C and left to equilibrate for 5 min. Finally the vials were measured for gelation by tilting them at 90° degree exhibiting no longer flow upon leaning to 90° degree angle [24]. For the gelation time analysis the CPNL was agitated continuously by using a magnetic stirrer at its gelation temperature. The time at which the movement of magnetic beads completely stopped was the time of gelation as the prepared nanogel was completely converted from sol to gel [25].

3.6. Rheological profile

Rheology plays vital role in stable preparation and effected delivery of gels, emulsion and solution. The prepared CPNL nanogel was evaluated for its rheological characteristics by using Rheometer (Anton paar) at 25 °C, 30 °C, and 35 °C of temperature [26].

3.7. Thermal analysis

The thermal assessment of CPNL nanogel was carried out by evaluating the thermogram attained by using the TG and DTA device (9SIITGDTA 6200 EXSTAR, USA) [27].

3.8. In-vitro drug release profile

The *in-vitro* drug release pattern of prepared CPNL was performed by employing the dialysis bag method using a shaking incubator at 100 rpm. Saline phosphate buffer with pH 4, 5, 6 and 7 was used as a dissolution medium so as to imitate the skin environment. The melanoma cell has pH range of 5.5–6.5 due to accumulation of lactic acid, whereas the normal melanin pH is exactly 7.4. Different pH range was provided to deeply evaluate the effect of nanogel on normal melanin cell [28]. Each dialysis bag (pore size: 12kD; sigma chemical co., St. Louis MO) were laden with about 10 ml of nanogel previously filtered through the Sephadex Column G-50. The *in-vitro* volume and temperature of dissolution medium were 50 ml, and 37 °C, respectively. At scheduled time 5 ml of the sample was withdrawn and substituted with the same volume of fresh media. The obtained sample was then filtered and evaluated for drug content at 304 nm against blank using UV–Visible spectrophotometer [29]. Mean results from triplicate measurements along with standard deviation were reported.

3.9. Blood compatibility analysis

3.9.1. Hemolysis assay—The hemolysis assay was carried out in CPNL nanogel using fresh human blood. About 2 ml of ACD solution (acid citrate dextrose) was added to 1.5 ml of blood sample and incubated for 1–2 h at 37 °C of temperature (100 µl of blood sample concentration ranging from 0.2 to 1.0 mg/ml). The incubated sample was centrifuged at 5000 rpm for 3–5 min to obtain plasma. The attained plasma was supplemented with 1 µl of 0.01% sodium bicarbonate solution [30]. The sample was then scanned at 450, 380 and 415 nm. The plasma hemoglobin was measured by employing equation:

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

For the smear slide preparation of blood and evaluation of CPNL, “*push and wedge*” coverslip method was used. Under this method, a drop of CPNL treated blood was placed on the slide at one end with the help of the pipette. The smear was spread by using spreader slide placed on the blood drop perpendicularly at an angle of 45°. The blood drop was then spread consistently on the base slide evading any shadowing and patchy width. The smear was then carefully air dried for 30 min for appropriate fixation. The fixed smear was then stained with Leishman’s stain (polychrome methylene blue and eosin) and baffled for about 15 min for cell picking. Finally, the slide was washed with running water and screened under the fluorescent microscope at 100× and 40× resolution lens (Olympus, Japan) [31].

3.9.2. Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) assessment—The Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) evaluation was employed for the demonstration of coagulation effect of CPNL. Fresh blood was collected in 10 ml ACD containing tubes and was centrifuged at 5000 for 5 min at 25 °C to obtain platelets poor plasma (PPP). 1000 µl of PPP was assorted with 0.1 µl of sample and incubated at 37 °C for 25 min [32]. After incubation the PT and APTT were evaluated by employing coagulation analyzer reagent kit (CK Prest and Fibriprest, Diagnosti casatgo, France).

3.10. Cytocompatibility analysis

3.10.1. Cell culture and seeding—Human Keratinocyte cell (HaCaT) line was obtained from NCCs Pune and was preserved in Dulbecco's modified Eagles Medium (DMEM). The cell line was then augmented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin, and 100 µg/ml streptomycin (PAA Laboratories GmbH, Austria) antibiotic solution. The HaCaT cell line was harvested in tissue culture flasks (75 cm²) and preserved at 5% CO₂ atmosphere at 37 °C. After reaching the 90% Confluency, the cells were trypsinized with 0.25% trypsin EDTA solution (Sigma, USA) [33]. For qualitative cell uptake and apoptosis study the HaCaT cells were seeded in 6 well plate (Costars, Corning Inc., NY, USA) as a density of 50,000 cells/well. Furthermore, MTT assay was also engaged to conclude the viability of the HaCaT cell line by seeding 10,000 cells/well in 96 well cell culture plates (Costars, Corning Inc. NY, USA) [34].

3.10.2. Cell uptake assay by CLSM—The advanced cell uptake and distribution studies were executed by using confocal laser microscopy (CLSM) for understanding the distribution capability of CPNL using HaCaT cell lines. The cells were incubated with CPNL (equivalent to 1 µg/ml) for 3 h. After incubation, the media comprising the CPNL was washed with Hanks buffered salt (HBS) solution (PAA Laboratories GmbH, Austria) three times and were detected under CLSM (Olympus FV1000) [35].

3.10.3. MTT assay—MTT assay was employed to analyze the cell cytotoxicity of the developed CPNL in HaCaT cell line. Briefly, HaCaT cell line was seeded in 96 well plates and incubated with media containing free capecitabine and CPNL (equivalent concentration of 0.1, 1, 10 and 20 µg/ml), negative control (cells treated with blank media) and positive control (Triton X-100). After 24 h of incubation, the media encompassing the samples were enunciated and cells were washed with HBSS three times. Thereafter, 150 µl of the MTT solution (500 µg/ml in PBS) was added to each well and re-incubated for 4 h. After 4 h the MTT solution was carefully articulated and the formazan crystals were then dissolved in 200 µl of DMSO. The optical density (OD) of the resultant solution was then screened at 570 nm using an ELISA plate reader (Bio Tek, USA) [36].

3.10.4. Apoptosis analysis—The cytotoxic efficiency of free drug Capecitabine and CPNL were assessed by estimating their competence to provoke apoptosis in HaCaT cell line. The Annexin V apoptosis assay is based on the Phosphatidyl serine exposure on the outer layer of the plasma membrane and its interaction with Annexin V. Briefly; HaCaT cells were seeded in the 6 well cell culture plate and kept for overnight at 37 °C and 5% CO₂. Then the media was aspirated and replaced with media containing free drug Capecitabine and CPNL (equivalent to 10 µg/ml) and incubated for 6 h. After incubation the media was enunciated and the cells were washed with HBSS three times and treated with Annexin V Cy3.18 (AnnCy3) and 6-carboxyfluorescein di-acetate (6-CFDA) Annexin V Cy3™ Apoptosis Detection kit, Sigma. USA). HaCaT cells were then observed under CLSM under green and red channels for 6-CFDA and AnnCy3, respectively. Furthermore, Apoptosis index, i.e., the fluorescence intensity ratio of red (a measure of apoptosis) and green (a measure of viability) channel was also assessed. The fluorescence strength in the images was evaluated *via* Image j software (U.S. National Institutes of Health, Bethesda,

Maryland, USA). The apoptotic Index (AI) can be calculated by dividing the percentage of apoptotic cells (annexin+) by the total percentage of cells in the sample (apoptotic [annexin +] plus no apoptotic cells [annexin-]). The following formula was used to calculate the apoptotic index:

$$AI = \%AV^{+}C / (\%AV^{+}C + \%AV^{-}C)$$

where %AV⁺C represent the percentage of annexin V positive cell and % AV⁻C represent the percentage of annexin V negative cells [37].

3.11. Ex-vivo skin permeation studies

For *ex-vivo* skin permeation evaluation, porcine skin was employed for the comprehensive determination of the penetration capability of CPNL. The porcine tissue is found to be the best model to mimic the human skin in comparison to the other species, for skin penetration studies. The permeation studies were carried out by using Franz diffusion (FD) cell with diffusion area of 3.3 cm² and volume 60 ml using porcine tissue [38].

3.11.1. Skin penetration analysis—The porcine tissue (pig ear) was obtained from the local slaughter house. The tissue was then sliced into the optimal size by using a surgical scalpel (DISPO VAN, Hindustan syringes & medical devices, Faridabad, India) prior to the deep cleaning and removal of hair. The skin penetration efficiency of the free capecitabine drug solution (FCDS) and CPNL were evaluated by observing the samples under a UV lamp prior tagging with Rhodamine 123 dye. For the skin permeation assay, the porcine tissue was sandwiched between the donor and acceptor compartment of the FD cell. About 1 ml tagged FCDS and CPNL was added in the donor compartment. The PBS solution (pH 5) at temperature 37 °C was incessantly stirred at 300 rpm serving as acceptor compartment dissolution media. The test samples were withdrawn after 24 h and equivalent amount of the PBS solution was replaced to maintain optimum sink condition. The collected test samples were then analyzed for drug released by UV spectroscopy method [39].

3.11.2. Drug concentration-depth profile—The amount of drug retent within the porcine tissue was evaluated by collecting the skin from the FD cell after the 24 h experimental exposure. The samples treated tissue was carefully washed with PBS solution (pH 5) and cut into 50 µm size using cryotome. The tissue was then divided and designated into 3 layers as upper, middle and lower epidermal layers. The retained drug from the tissue was extracted by adding 5 ml ethyl alcohol, stirred for about 10 h and centrifuged at 5000 rpm for 20 min. The supernatant was collected and filtered *via* 0.2 mm syringe filter and analyzed for the drug content by UV spectrophotometry method [40].

3.11.3. Fluorescent imaging (FI)—The tissue distribution and localization potential of FCDS and CPNL were analyzed by Fluorescent Imaging (FI). The tissue obtained post treatment with Rhodamine 123 tagged FCDS and CPNL was collected and washed carefully with a PBS solution (pH 5). The tissue was then sectioned by a cryotome to obtain 5 µm size pieces prior to group in 3. Finally, the sectioned tissues were treated with xylene to eliminate the water traces and placed in the clean glass slide for the analysis of drug retention. The

drug retention efficiency of both samples at different epidermal depth was analyzed by using fluorescent microscope (Olympus, japan) [41].

3.11.4. Histological analysis—For the histological analysis, the experimental tissue sample was collected, washed thoroughly with a PBS solution (pH 5) and fixed by using 10% w/v formaldehyde solution. After treatment with formaldehyde, the tissue were processed to eradicate the formaldehyde and sectioned by using microtome. Finally, the sectioned tissues were preserved and stained with hematoxylin and eosin dye and visualize under the microscope (Olympus, japan) [42].

3.12. Statistical analyses

The values were expressed as mean + SD. Statistics. Statistical analysis of the data was performed *via* one-way analysis of variance (ANOVA) using origin software; a value of $p < 0.01$ was considered significant, ($n = 3$) [43].

4. Results & discussion

4.1. Synthesis of skin permeating nanogel

The CPNL was synthesized by an ion-gelation method using sodium tripolyphosphate (TPP) as cross-linker with Pluronic 127 as solvent system and Transcutol as penetration enhancer. The ionic bonding between the core cationic amino group on chitosan backbone with amiable anionic phosphate group of TPP results in the robust chemical crosslinking portent [44]. The copolymer of propylene oxide and ethylene oxide (Pluronic 127) is a hydrophilic copolymer. It produces mono-molecular micelles at low concentration ($<5\text{--}10\%$ w/v) and multi-molecular clusters at higher concentration ($>15\%$ w/v). The Pluronic 127 at gel phase exhibits a hydrophobic central core, exposing hydrophilic polyoxyethylene chain amiable to the external environment. Micelle formation of Pluronic-127 occurs above critical micellar concentration in the appropriate solvents system at optimal temperature. Above critical micellar concentration the micelles achieve a lattice configuration and form gel [45]. The Transcutol at an optimized concentration of 24% v/v leads to the better penetration *via* stratum corneum and diffusion *via* lipid matrix of skin. The amalgam of Pluronic 127 and Transcutol forms a stable nanogel network avoiding local cellular toxicities and edema. Chitosan demonstrated the cationic nature which plays a key role in ionic bonding with tumor cell. The tumor cell membrane exhibited anionic charge due to the accommodation of lactic acid, leading in lowering of cell pH from 7.5 (pH of normal cell) to 5.5–6.5. Ionic interaction between cationic chitosan and anionic tumor cell facilitates significant covalent bonding, leading in controlled release of capecitabine at the tumor site.

4.2. Particle size, zeta potential and morphology

The results obtained from the zeta sizer analysis showed diverse size range of CPNL between 100 and 150 nm. The smaller size of the CPNL displayed optimum entrapment of capecitabine within the polymer (Chitosan) matrix due to the formulation and process optimization. The surface charge of CPNL nanogel was +41.21 mV demonstrated cationic nature of CPNL. The zeta potential befalls between 40 and 60 mV exhibiting noteworthy stability for efficient transdermal delivery. The entrapment efficiency was 71% exhibiting

decent drug encapsulation for desired therapeutic efficiency. The pH of CPNL was 5.9 ± 0.21 which play a vital role in acid activation and served as a driving force for the competent transdermal delivery at the tumor site. The pH triggered mechanism act as the key element for the onsite degradation of the polymer matrix. The pH responsive characteristics discharge capecitabine at targeted site in slightly acidic environment (pH 5.5–6.5). This acid activation mechanism enhanced the drug release at a controlled rate leading to desired therapeutic potential and elimination of local cellular toxicity.

4.3. Transmission electron microscopy (TEM)

The Transmission Electron Microscopy (TEM) evaluation exhibited very distinct particles size portraying spherical and nonabrasive boundaries of nanoparticles. The size exhibited by TEM is between 120 and 150 nm (Fig. 2a & b) authenticating DLS and zeta sizer outcomes significantly. The slight aggregation of nanoparticles was observed is due to the hydrophilic nature of polymer having a tendency to adsorb and get agglutinate. The TEM outcomes displayed suitable nano carrier system for the effective transdermal delivery, revealing decent skin infiltration appearance of CPNL.

4.4. Scanning electron microscopy (SEM)

The SEM images validated and confirmed the outcomes of TEM and DLS analysis exhibiting fine morphology and surface texture of CPNL with optimal spherical shape (Fig. 2c & d). The SEM pictures visualized clear and sharp spherical texture of CPNL. Slight cluster of particles was found due to the hydrophilic nature of Chitosan displaying tendency of adsorption and agglomeration [16]. The SEM outcomes displayed 100–150 nm size range, authenticating the TEM evaluation and confirming the ideal transdermal delivery characteristics of CPNL.

4.5. DLS analysis

The Dynamic Light Scattering (DLS) results showed sharp and detailed size distribution pattern of CPNL. The nanosize of CPNL showed an enhanced delivery to the dermal area and personified the size of cells and its micro-environment. The DLS investigations displayed varied size distribution of CPNL between 120 and 160 nm and exhibited PDI of 0.213 ± 0.15 (Fig. 2e & f). The DLS outcomes exhibited significant stability and optimal size distribution between 100 and 400 nm. This facilitates better diffusion of CPNL across the skin barricades leading to desired therapeutic potential at targeted site. The DLS outcomes significantly validated the results of zeta sizer endorsing CPNL for stable and ideal nanocarrier for effective transdermal delivery.

Overall, the operational potential of any transdermal delivery symbiotically depends on the particle size and morphology. The ideal size range falls between 200 and 400 nm for efficient transdermal diffusion *via* skin. The spherical particle shape shows better transportation and onsite retention of cytotoxic agent *via* endocytosis mechanism [44]. The particle size of CPNL ranges between 100 and 150 nm with spherical shape and smooth morphology by TEM, SEM and DLS analysis. Therefore, it can be forthrightly state out that CPNL exhibited ideal characteristics for the operative transdermal delivery in clinical platform.

4.6. Gelation time studies

Gelation time denotes the time required to convert the solution to gel at specific temperature and time by developed nanogel formulation. The CPNL showed gelation time of 39 ± 1.22 at 22% w/v of Pluronic 127 concentration at 28–30 °C and convert back to solution at 8 °C. The gelation time showed negligible deviation w.r.t temperature displaying stable transdermal delivery of CPNL. As the concentration of PF 127 increases the gelling time and viscosity of CPNL increase symbiotically [24]. Therefore, optimization of CPNL for gelling physiognomies plays a key role to obtain the stable and effective transdermal delivery system for enhanced therapeutic potential.

4.7. Rheological profile

The CPNL showed a discrete rheological configuration at different temperature. The viscosity was found 3315 ± 4.55 cps at 32 °C showing stable gelling system for operative transdermal delivery. The CPNL exhibited different rheological behavior at 25–28 °C exhibiting viscosity of 3050 ± 2.98 and found stable at 30–34 °C (Fig. 3a). The varied viscosity displayed mixed behavior of Pluronic 127 in the formation of multi-molecular micelles. The unconventionality in the rheological pattern at different temperature is due to the hydrophilicity of nanogel. The rheogram vividly portrayed that as the temperature increases from 25 °C to 30 °C enhancement of viscosity and shear rate observed [46]. The viscosity results showed a symbiotic relationship between viscosity and temperature. This is due to the existence of hydrophilic Chitosan backbone in PF 127 gel network resulting in elevation of viscosity and elasticity of CPNL.

4.8. TG and DTA analysis

The thermal degradation evaluation of CPNL is varied with increase in temperature. The degradation of CPNL is biphasic in nature and falls into two stages w.r.t temperature. The two stage degradation exhibited enhanced thermal stability and compression of nanogel matrix. The degradation was meager and slow at initial stage exhibiting 15–25% nanogel decomposition between 40 °C to 90 °C of temperature. As the temperature increases above 170 °C the rate of decomposition was enhanced by 75% of nanogel degradation. On further elevation (>200 °C) the degradation was stable with constant loss of polymer weight w.r.t. time and temperature (Fig. 3b). The DTA and TG pattern exhibited enhanced thermal stability of CPNL exhibiting exothermic peak in the thermogram. This thermal stability is directly influenced by strong covalent bonding between NH_2 and OH^- of Chitosan and capecitabine leading to optimum entrapment inside CPNL [18]. This ionic bonding resulted in effective transdermal delivery and noteworthy intracellular transport at the targeted site avoiding early nanogel degradation at unwanted cellular area.

4.9. pH responsive drug release profile

The *in-vitro* drug release pattern of CPNL was carried out by employing dialysis bag in phosphate buffer solution (PBS) at pH 4, 5, 6 and 7 at 37 °C. The drug release pattern at different pH was nearly identical at slightly acidic pH. The biphasic drug release behavior was observed with initial bursting of nanoparticles in early 1–6 h followed by slow and sustained release in 24 h (Fig. 3c). The drug release mechanism from nanogel was totally

governed by diffusion constant, molecular weight and degree of de-acetylation of Chitosan. The early bursting of CPNL was due to the hydrophilic nature of Chitosan, exhibiting early polymer erosion and elevated water absorption through hydrolysis. The degradation mechanism was also depends on covalent bonding of nanoparticles and de-acetylation degree of Chitosan. It has been predict out that higher the degree of de-acetylation of polymer, slower will be the drug release and degradation of CPNL. The Chitosan exhibited 70% degree of de-acetylation resulted in the biphasic drug release by CPNL in 24 h. The CPNL showed early release of about 15–25% at pH 7 in 24 h, eventually displaying the non-viability towards the normal melanin cells. The sustained drug release was recorded at slightly acidic environment pH (5 & 6) in 24 h imitating tumor microenvironment. The nanogel showed insufficient drug release at pH 7 signifying non viability on normal melanin cells. Significant drug release was observed at acidic pH 4 and slightly acidic pH (5 & 6) displaying pH responsive release property of CPNL in 12–24 h. The total drug release followed the Higuchi's kinetics model with controlled drug release due to the de-acetylation degree of Chitosan polymer.

4.10. Blood compatibility assay

4.10.1. Hemolysis evaluation—The hemolysis effect of transdermal formulation plays vital role in generating an effective and safe nanoformulation at clinical platform. The hemolysis effect greatly influenced erythrocyte morphology, ionic balance and membrane fluidity in blood. It also reduced oxygen carrying capacity of RBCs (Red blood cells) due to insufficient blood supply to the heart leading to cardiotoxicity [47]. We developed transdermal CPNL nanogel for the onsite delivery of cytotoxic drug (Capecitabine) against skin cancer at the controlled rate. Negligible hemolysis was shown by CPNL at different concentration (Fig. 3d–f) when equated with positive control. The hemolysis displayed by CPNL at 0.2–0.6 mg/ml concentration revealed negligible hemolysis. Slight hemolysis was observed at 0.8 and 1.0 mg/ml concentration, signifying linear relationship between nanogel concentration and hemolysis ratio. The hemolysis ratio of CPNL was found to be 3.69% exhibiting biological safe margin. The ISO/TR 7406 ethical guidelines allows 5% critical safe hemolysis ratio for biomaterials [48]. The smear slide of CPNL revealed negligible ruptures of RBCs at all concentration. Significant hemolysis was observed in positive control slide with clear lysis of RBCs compared to negligible hemolysis in negative control. The clear plasma and RBCs existence at concentration 0.2–1.0 mg/ml revealed negligible hemolysis. On the other hand positive control vial showed opaque red color plasma, indicating complete hemolysis and leaching of heam and other contents. Therefore, the developed CPNL is innocuous and revealed negligible hemo-toxicity index towards blood.

4.10.2. PT & APTT assay—For the advanced biocompatibility evaluation, coagulation assay (PT and APTT) was carried out for the CPNL nanoformulation. The PT investigation determined the extrinsic pathway and the APTT assay demonstrated the intrinsic pathway of blood coagulation phenomenon. Both the compatibility elements envisaged the biosafety of nanogel. The PT and APTT time displayed by the CPNL was 15.3 ± 0.21 and 33.6 ± 0.67 respectively, exhibiting the biosafety level of CPNL. The normal blood (devoid of nanogel and drug) exhibited PT and APTT time of 12.1 ± 1.27 and 30.11 ± 2.85 s respectively, exhibiting identical coagulation time with CPNL. The outcomes of PT and APTT

evaluations showed, CPNL nanogel do not alter the intrinsic and extrinsic pathway of blood coagulation. Therefore, the biosafety margin of CPNL nanogel was found to be significant and biologically safe for operative transdermal delivery.

4.11. Cytotoxicity studies

4.11.1. Cell uptake & distribution assay by CLSM—The Confocal Laser Scanning Microscopy (CLSM) displayed significant uptake and distribution of CPNL evaluated on HaCaT cell lines. The CLSM outcomes exhibited noteworthy internalization and distribution of CPNL. The fluorescent intensity demonstrated sharp ratification of CPNL around the nucleus region of cells (marked red arrow). The cellular uptake images of CPNL revealed large patches occurrence with expanded sign of fluorescence at the outer cell surface. The intense fluorescence signals displayed by CPNL on HaCaT cell line are the sign of vesicular localization of nanogel demonstrating endocytic pathway progression. The native vesicular uptake also exhibiting pinocytosis mechanism and non-phagocytosis phenomenon of CPNL on HaCaT cell line (Fig. 4a–d). Therefore, the CLSM outcomes significantly demonstrated size dependent internalization uptake and distribution of CPNL.

4.11.2. Cytotoxicity assessment by MTT assay—The MTT assay demonstrated that free drug Capecitabine and CPNL are cytocompatible at all concentration employed in cytotoxicity measurement against the HaCaT cell line. The MTT results noteworthy showed that CPNL exhibited sharp toxicity towards the HaCaT cell when compared with normal control. The cytotoxicity of CPNL nanogel towards HaCaT cell was established statistically noteworthy compared to normal control ($p < 0.01$) (Fig. 4e). The plain Capecitabine showed optimum toxicity towards HaCaT cell due to direct viability of free drug. The Capecitabine inhibits the metabolic pathway and converts itself to 5-Fluorouracil leading in apoptosis initiation in HaCaT cell. The MTT assay noteworthy exhibited conservation of cell integrity devoid of any alteration cell microenvironment processes. The significant cytotoxicity of CPNL leads to better development of the alternate nanocarrier over the existing one for the enhanced anticancer potential *via* transdermal route.

4.11.3. Apoptosis assay—The Apoptosis evaluation employing free drug capecitabine and developed CPNL demonstrated noteworthy apoptosis at all concentrations (Fig. 4f & g). The free capecitabine displayed inherent apoptosis while CPNL showed both intrinsic (mitochondrial) and extrinsic (death receptor) apoptosis pathway. The capecitabine induces apoptosis by targeting on the G2 phase leads to mitochondrial apoptosis phenomenon. In the extrinsic apoptosis pathway, it is assumed that capecitabine act as a death activator by encouraging cell surface receptor. Activating cell surface receptor leads to the stimulation of caspase 8 and caspase cascade. The apoptosis index of plain drug was 0.51 whereas the CPNL exhibited the apoptosis index of 0.82. The CPNL showed potent apoptosis action (Fig. 4g, ii) (marked yellow arrow) compared to plain Capecitabine (Fig. 4f, ii) which is nearly two folds more and found significant ($*p < 0.01$). The main reason behind enhanced apoptosis of CPNL over plain drug is the nanosized molecules, causing rapid onsite transportation and sufficient distribution in HaCaT cell line. Also, the cationic charge of CPNL enabled noteworthy apoptosis by diffusing through stratum corneum or hair follicle pathways by endocytosis. This leads to the efficient ionic binding of cationic CPNL to the

anionic charge tumor cell membrane. The ionic interaction enabled the effective degradation of Chitosan matrix and Capecitabine release at the tumor site without avoiding local cellular toxicity.

4.12. Ex-vivo skin permeation evaluation

4.12.1. Skin penetration by steady state flux—The *ex-vivo* permeation results elaborated enhanced diffusion of CPNL by Franz diffusion cell employed against porcine tissue compared to free capecitabine drug solution (FCDS) under a UV lamp (Fig. 5a & b). The UV spectrophotometry analysis illustrated noteworthy diffusion of CPNL depicting 70% drug release compared to FCDS in the 24 h experiment. The flux rate of CPNL compared to FCDS is 3 folds better and found significant ($p < 0.01$). There exists a symbiotic relationship between diffusion constant (D) and steady state flux (J). The elevation in D is directly proportional to the J due to enhanced disruption of corneocytes leading to the better steady state flux (J). The transcellular and intracellular transport *via* hair follicle pathway and sweat glands pathway respectively, plays a vital role in efficient penetration and diffusion of CPNL. Therefore, the CPNL system showed significant and enhanced penetration potential compared to FCDS when analyzed by *ex-vivo* penetration assay.

4.12.2. Concentration-depth profile—The CPNL concentration depth profile showed very sharp uptake and retention of capecitabine from nanogel matrix in the skin epidermal layer. The experimental exposure of porcine tissue confirmed significant diffusion of capecitabine from CPNL when compared with FCDS. The concentration depth analysis validated steady state flux depicting enhanced penetration and retention efficacy of CPNL at different epidermal layer. The fluorescent microscopic images showed noteworthy diffusion and retention at upper epidermal layer compared to middle and lower layer (Fig. 5c). The penetration and retention potential of CPNL at all epidermal layers was 3 fold better than FCDS and found significant ($p < 0.01$). The ionic interaction between positively charged CPNL with negatively charged cell membrane caused efficient binding to tumor cell surface. The pH triggered mechanism and surface decoration by Transcutol enabled onsite drug release producing novel alternate transdermal therapy in skin cancer.

4.12.3. Fluorescent imaging evaluation—The *ex-vivo* CPNL fluorescent images of various epidermal layers elaborated enhanced permeation and diffusion efficacy at various epidermis layers compare to the FCDS. The fluorescent strength of CPNL treated porcine tissue displayed sharp intensity in upper epidermis, which is twofold better compared to the middle and lower epidermis layer. The fluorescent strength by FCDS exhibited meager signals in all three epidermal layers. The fluorescent strength at middle and lower epidermal layer of CPNL treated tissue was also noteworthy compared to the FCDS treated tissue at middle and lower epidermal layer (Fig. 6i–vi). On inter-comparison of fluorescent strength, middle and lower epidermal layer of CPNL treated porcine tissue exhibited identical intensity to upper epidermal of FCDS treated tissue (Fig. 6I–VI). The inter-comparison fluorescent intensity of both the treated tissue significantly demonstrated the enhanced permeation and retention of CPNL in all epidermal layers. The fluorescent microscopy images of epidermal layers demonstrated significant penetration potential of CPNL on

transdermal delivery. Overall, the capecitabine encapsulated in Chitosan core showed optimal penetration and controlled release physiognomies mediated by Transcutol. This results in the fine uptake and decent distribution of capecitabine at the tumor site, offering promising alternate transdermal therapy against skin cancer.

4.12.4. Histology profile—The *ex-vivo* histology profile showed noteworthy permeation potential of CPNL compared to FCDS solution. The histology evaluation of CPNL displayed significant disruption of the squamous layer with the slackening of lipid bilayers compared to FCDS treated tissue. The lipid bilayer disruption of CPNL treated tissue is higher due to the lipophilic nature of capecitabine (Fig. 6a–c). Stratum Corneum (SC) act as the initial check for any formulation delivered transdermally [6]. The disruption of SC validated the better penetration strength of CPNL over FCDS without generating any annoying eruptions or erythema and swelling of SC. The *ex-vivo* histological assay confirmed stable flux and augmented penetration potential of CPNL. The enhanced penetration is greatly mediated by surface decoration by Transcutol in all epidermal layers of porcine tissue (analyzed by one way ANNOVA test). The histology results significantly confirmed the enhanced diffusion, skin penetration and retention potential of CPNL. Overall, the *ex-vivo* outcome opens the novel alternative transdermal nanotherapy against skin cancer with low dose interval and negligible cellular toxicity.

5. Conclusion

In search of topical alternative against skin cancer, an innovative system has been discovered and contrived utilizing existing synthesis techniques and established antimetabolite. The CPNL was surface decorated by Transcutol for enhanced penetration, exhibiting controlled release of capecitabine at defined entrapment efficiency against skin cancer. The concluding CPNL was synthesized by optimizing the process and formulation parameters for obtaining stable and nanosized particles. Optimized CPNL possess better penetration and transportation across SC and diffuses *via* skin lipids for enhanced retention and distribution at the tumor site. The current *in-vitro* and *ex-vivo* outcomes employing porcine tissue discovered the effectiveness and transdermal viability of CPNL in the management of skin cancer. Overall CPNL is discovered as an encouraging alternative transdermal contender with remarked clinical translational efficiency.

Acknowledgements

The research work was supported by Indian Council of Medical Research, New Delhi, India as Senior Research Fellowship to Prashant Sahu with Ref No. 45/28/2014-Nan/BMS dated 21/03/2016. We pay our acknowledgement to Sophisticated Instrumentation Analysis Laboratory, Dr. Harisingh Gour Central University, Sagar, M.P. India for TEM, SEM and other facilities. Thanks to SRL laboratory, Gurgaon, India for Hemolysis, PT & APTT studies along with providing us the histology study facilities. We pay our sincere greets to Centre for Pharmaceutical Nanotechnology, department of Pharmaceutics, National institute of Pharmaceutical Educational and research (NIPER), Mohali, Punjab, India for pharmacokinetic assessment work. Dr. Sushil K. Kashaw wish to acknowledge the University Grants Commission, New Delhi for the award of Raman Postdoctoral Research Fellowship in Use-inspired Biomaterials & integrated Nano Delivery (U-BiND) Systems Laboratory, Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, Detroit, MI, USA.

References

- [1]. Smith KJ, Germain M, Skelton HG, Squamous cell carcinoma in situ Bowen's disease in renal transplant patients treated with 5% imiquimod and 5% 5-fluorouracil therapy, *Dermatol. Surg* 27 (2001) 5611–5614.
- [2]. Epstein E, Does intermittent “pulse” topical 5-fluorouracil therapy allow destruction of actinic keratoses without significant inflammation? *J. Acad. Dermatol* 38 (1998) 77–80.
- [3]. Pearlman DL, Weekly pulse dosing: effective and comfortable topical 5-fluorouracil treatment of multiple facial actinic keratoses, *J. Am. Acad. Dermatol* 25 (1998) 665–667.
- [4]. El Shabrawi-Caelen L, Kerl H, Cerroni L, Melanin-A: not a helpful marker in distinction between melanoma in situ on sun-damaged skin and pigmented actinic keratosis, *Am. J. Dermatopathol* 26 (2004) 364–366 (33). [PubMed: 15365366]
- [5]. Beltraminelli H, Shabrawi-Caelen L, Kerl H, Cerroni L, Melan-apositive “pseudomelanocytic nests”: a pitfall in the histopathologic and immunohistochemical diagnosis of pigmented lesions on sundamaged skin, *Am. J. Dermatopathol* 31 (2009) 305–308. [PubMed: 19384076]
- [6]. Sahu P, Das D, Mishra VK, Kashaw V, Kashaw SK, Nanoemulsion: a novel eon in cancer chemotherapy, *Mini-Rev. Med. Chem* 17 (2017) 1778–1792. [PubMed: 26891931]
- [7]. Das D, Sahu P, Mishra VK, Kashaw SK, Nanoemulsion-the emerging contrivance in the field of nanotechnology, *Nanosci. Nanotech. Asia* 2 (2017) 1–22.
- [8]. Peer D, Karp J, Hong S, Farokhzad O, Margalit R, Langer R, Nanocarriers as an emerging platform for cancer therapy, *Nat. Nanotechnol* 2 (2007) 751–760. [PubMed: 18654426]
- [9]. Davis ME, Chen ZG, Shin DM, Nanoparticle therapeutics: an emerging treatment modality for cancer, *Nat. Rev. Drug Discov* 7 (2008) 771–782. [PubMed: 18758474]
- [10]. Farokhzad O, Langer R, Impact of nanotechnology on drug delivery, *ACS Nano* 3 (2009) 16–20. [PubMed: 19206243]
- [11]. Alexis F, Pridgen E, Molnar LK, Farokhzad OC, Factors affecting the clearance and biodistribution of polymeric nanoparticles, *Mol. Pharm* 5 (2008) 505–515. [PubMed: 18672949]
- [12]. Griffin DR, Weaver WM, Scumpia PO, Di Carlo D, Segura T, Accelerated wound healing by injectable microporous gel scaffolds assembled from annealed building blocks, *Nat. Mater* 14 (2015) 737–744. [PubMed: 26030305]
- [13]. Chacko RT, Ventura J, Zhuang J, Thayumanavan S, Polymer nanogels: a versatile nanoscopic drug delivery platform, *Adv. Drug Deliv. Rev* 64 (9) (2012) 836–851. [PubMed: 22342438]
- [14]. Eckmann D, Composto R, Tsourkas A, Muzykantov V, Nanogel carrier design for targeted drug delivery, *J. Mater. Chem. B* 2 (46) (2014) 8085–8097. [PubMed: 25485112]
- [15]. Bhattarai N, Gunn J, Zhang M, Chitosan-based hydrogels for controlled, localized drug delivery, *Adv. Drug. Deliv. Rev* 62 (1) (2010) 83–99. [PubMed: 19799949]
- [16]. Brunel F, Véron L, Ladaviere C, David L, Domard A, Delair T, Synthesis and structural characterization of chitosan nanogels, *Langmuir* 25 (16) (2009) 8935–8943. [PubMed: 19572536]
- [17]. Keawchaon R, Yoksan, Preparation, characterization and in vitro release study of carvacrol-loaded chitosan nanoparticles, *Colloids Surf., B: Biointer-faces* 84 (2011) 163–171.
- [18]. Kabiri K, Omidian H, Hashemi S, Zohuriaan-Mehr M, Synthesis of fast-swelling superabsorbent hydrogels: effect of crosslinker type and concentration on porosity and absorption rate, *Eur. Polym. J* 39 (7) (2003) 1341–1348.
- [19]. Zhang X, Malhotra S, Molina M, Haag R, Micro-and nanogels with labile crosslinks—from synthesis to biomedical applications, *Chem. Soc. Rev* 44 (7) (2015) 1948–1973. [PubMed: 25620415]
- [20]. Sultana F, Imran-Ul-Haque M, Arafat M, Sharmin S, An overview of nanogel drug delivery system, *J. Appl. Pharm. Sci* 3 (8 Suppl 1) (2013) S95.
- [21]. Sarika P, Kumar PA, Raj DK, James NR, Nanogels based on alginic aldehyde and gelatin by inverse miniemulsion technique: synthesis and characterization, *Carbohydr. Polym* 119 (2015) 118–125. [PubMed: 25563951]

- [22]. Sahu P, Kashaw SK, Kushwah V, Sau S, Jain S, Iyer AK, pH responsive biodegradable nanogels for sustained release of bleomycin, *Bioorg. Med. Chem* 25 (2017) 4595–4613. [PubMed: 28734664]
- [23]. Sahu P, Kashaw SK, Sau S, Iyer AK, Stimuli-responsive bio-hybrid nanogels: an emerging platform in medicinal arena, *Global J. Nanomed* 1 (2017) 1–3.
- [24]. Brunel F, Véron L, David L, Domard A, Delair T, A novel synthesis of chitosan nanoparticles in reverse emulsion, *Langmuir* 24 (20) (2008) 11370–11377. [PubMed: 18774829]
- [25]. Pujana MA, Pérez-Álvarez L, Iturbe LCC, Katime I, Water dispersible pH-responsive chitosan nanogels modified with biocompatible crosslinking-agents, *Polymer* 53 (15) (2012) 3107–3116.
- [26]. Das D, Sahu P, Kashaw V, Kashaw SK, Formulation and assessment of in vivo anti-inflammatory potential of omega-3-fatty acid loaded self emulsifying nanoemulsion, *Curr. Nanomed* 7 (2017) 47–58.
- [27]. Chaurasia A, Sahu P, Gajbhiye V, Improved anticancerous activity of Indian aloe loaded chitosan microspheres, *Int. J. Pharm. Arch* 3 (2013) 71–76.
- [28]. Lai H, Chen Q, Wu P, The core-shell structure of PNIPAM collapsed chain conformation induces a bimodal transition on cooling, *Soft Matter* 9 (2013) 3985–3993.
- [29]. Dowding PJ, Vincent B, Williams E, Preparation and swelling properties of poly (NIPAM) “minigel” particles prepared by inverse suspension polymerization, *J. Colloid Interface Sci* 221 (2000) 268–272. [PubMed: 10631030]
- [30]. Lele AK, Hirve MM, Badiger MV, Mashelkar RA, Predictions of bound water content in poly(N-isopropylacrylamide) gel, *Macromolecules* 30 (1997) 157–159.
- [31]. Vishwakarma APS, Vishwe A, Sahu P, Chaurasia A, Magical remedies of *Terminalia arjuna* Roxb, *Int. J. Pharma. Arch* 2 (2013) 189–201.
- [32]. Mueller E, Alsop RJ, Scotti A, Bleuel M, Rheinstädter MC, Richtering W, Hoare T, Dynamically cross-linked self-assembled thermoresponsive microgels with homogeneous internal structures, *Langmuir* 34 (2018) 1601–1612. [PubMed: 29261314]
- [33]. Williams G, Watts DC, Non-symmetrical dielectric relaxation behaviour arising from a simple empirical decay function, *Trans. Faraday Soc* 66 (1970) 80–85.
- [34]. Ngai KL, Dynamics of semidilute solutions of polymers and associating polymers, *Adv. Colloid Interf. Sci* 64 (1996) 1–43.
- [35]. Sahu P, Bhatt A, Chaurasia A, Gajbhiye V, Enhanced hepatoprotective activity of piperine loaded chitosan microspheres, *Int. J. Drug Dev. Res* 4 (2012) 259–262.
- [36]. Phillies GDJ, Richardson C, Ann Quinlan C, Ren SZ, Transport in intermediate and high molecular weight hydroxypropylcellulose/water solutions, *Macromolecules* (1993) 6849–6858.
- [37]. Yadav S, Sahu P, Chaurasia A, Role of *Cyamopsis tetragonoloba* against cisplatin induced genotoxicity: analysis of micronucleus and chromosome aberrations in vivo, *Int. J. Bio Innov* 2 (2013) 184–193.
- [38]. Sagnella S, Mai-Ngam K, Chitosan based surfactant polymers designed to improve blood compatibility on biomaterials, *Colloids Surf. B: Biointerfaces* 42 (2005) 147–155. [PubMed: 15833667]
- [39]. Das D, Chaurasia A, Sahu P, Mishra VK, Kashaw SK, Antihypercholesterolemic potential of omega-3-fatty acid concentrate in alloxan induced diabetic rodent, *Int. J. Pharm. Sci. Res* 6 (2015) 3634–3640.
- [40]. Sahu P, Kashaw SK, Jain S, Sau S, Iyer AK, Assessment of penetration potential of pH responsive double walled biodegradable nanogels coated with eucalyptus oil for the controlled delivery of 5-fluorouracil: in vitro and ex vivo studies, *J. Control. Release* 253 (2017) 122–136. [PubMed: 28322977]
- [41]. Gan D, Lyon LA, Synthesis and protein adsorption resistance of PEG-modified poly(N-isopropylacrylamide) core/shell microgels, *Macromolecules* 35 (2002) 9634–9639.
- [42]. Mohtashamian S, Boddohi S, Nanostructured polysaccharide-based carriers for antimicrobial peptide delivery, *J. Pharm. Investig* 47 (2) (2017) 85–94.
- [43]. Chaurasia A, Das D, Sahu P, Evaluation, assessment & screening of antidiabetic activity of *Stevia rebaudiana* leaves extract in alloxan-induced diabetic rat model, *J. Int. Res. Med. Pharma. Sci* 9 (2016) 107–112.

- [44]. Sahu P, Kashaw SK, Kushwah V, Sau S, Jain S, Iyer AK, pH responsive 5-fluorouracil loaded biocompatible nanogels for topical chemotherapy of aggressive melanoma, *Colloids Surf. B: Biointerfaces* 174 (2019) 232–245. [PubMed: 30465998]
- [45]. Vishwakarma APS, Vishwe A, Sahu P, Chaurasia A, Screening of hepatoprotective potential of ethanolic and aqueous extract of *Terminalia arjuna* bark against paracetamol/CCl₄ induced liver damage in Wistar albino rats, *Int. J. Pharma. Arch* 3 (2013) 1–8.
- [46]. Wang H, Zhang R, Zhang H, Jiang S, Liu H, Sun M, Jiang S, Kinetics and functional effectiveness of nisin loaded antimicrobial packaging film based on chitosan/poly (vinyl alcohol), *Carbohydr. Polym* 127 (2015) 64–71. [PubMed: 25965457]
- [47]. Gharsallaoui A, Joly C, Oulahal N, Degraeve P, Nisin as a food preservative: part 2: antimicrobial polymer materials containing nisin, *Crit. Rev. Food Sci. Nutr* 56 (8) (2016) 1275–1289. [PubMed: 25674671]
- [48]. Mangalathillam S, Rejinold NS, Nair A, Lakshmanan VK, Naira SV, Jayakumar R, Curcumin loaded chitin nanogels for skin cancer treatment via the transdermal route, *Nanoscale* 4 (2012) 239–250. [PubMed: 22080352]

Fig. 1. Schematic representation of CPNL synthesis technique, mechanism of action and drug release phenomenon at tumor site on transdermal delivery.

Fig. 2.

Demonstration of (a & b) TEM analysis of CPNL at 500 and 100 nm of resolution (c & d) SEM analysis of CPNL at 100 nm and 1 μm of resolution with cluster formation (e) DLS analysis and (f) histogram of particle size distribution of CPNL nanogel respectively (Mean $\pm$ SD, n = 3).

Fig. 3.

Demonstration of (a) Rheology analysis of CPNL w.r.t temperature (b) DTA and TG analysis of CPNL at different temperature (c) pH responsive drug release in PBS buffer (d) hemolysis evaluation at various concentration of CPNL treated blood (0.2–1.0 mg/ml), (e) visual analysis of hemolysis in blood vials at different concentration of CPNL treated blood (0.2–1.0 mg/ml) (f) microscopic evaluation of CPNL (1.0 mg/ml concentration) treated blood smear slide stained with 1% leishman's dye at 100× and 40× lens resolution using fluorescent microscope (Olympus, Japan) displaying no lysis of RBCs in blood treated nanogels system. (Olympus, Japan), each data points were repeated in triplicates (n = 3) and presented as Mean ± Standard Deviation (S.D).

Fig. 4.

Elaboration of cell uptake profile by confocal microscopy, images (a–d) showing cell uptake of CPNL nanogel on HaCaT cell line exhibiting under the green fluorescence channel along with differential interface contrast images, while image (e) demonstrating MTT assay of CPNL on HaCaT cell line by different samples at different concentration. Images (I) showing apoptosis assay of free drug capecitabine (10 μg/ml; 6 h incubation) and (II) showing apoptosis assay of CPNL nanogel (equivalent to 10 μg/ml and incubated for 6 h) against HaCaT cells; (i) green channel depicts the fluorescence from carboxy fluorescein (cell viability marker dye), (ii) red channel depicts fluorescence from Annexin Cy3.18 conjugate (cell apoptosis marker dye), (iii) overlay images of figure whereas, (iv) depicted differential contrast image of representative cells. The apoptosis index measured as ratio fluorescence intensity from the red channel to that of green channel. The fluorescence intensities of the images were measured using Image J software, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/> (n = 3, data expressed as average ± SE, *denotes p < 0.01).

Fig. 5.

Illustration of acceptor fluid collection from skin permeation experiment (a) image depicting CPNL and free Capecitabine drug solution (FCDS) under UV light (b) steady state flux displaying cumulative drug release of FCDS and CPNL permeation w.r.t time (c) skin penetration analysis depicting concentration of extracted drug at various epidermis layers of porcine tissue treated with FCDS and CPNL after 24 h of exposure (n = 3, data expressed as average $\pm$ SE, *denotes $p < 0.01$).

Fig. 6. Demonstration of skin localization of (i–vi) developed CPNL nanogel and (I–VI) FCDS at different epidermal layers of porcine tissue by fluorescent microscopy on 4 h incubation. Images (a–c) showing histology pictures of normal (untreated), FCDS treated and CPNL treated porcine tissues visualized at 100× magnification using light microscope (Olympus, Japan), $p < 0.01$, $n = 3$.