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Synthesis, characterization and safety evaluation of bilirubin nanoparticles as a topical formulation for wound healing

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Abstract
Nanotechnology is one of the emerging area of research interest in the present era. Nanomedicine has been developed for a number of diseases and has been tested in the different disease models. However, evaluating the safety of nanomedicine is an important aspect. In the present study, bilirubin nanoparticles were synthesized by our novel method and characterized for different parameters such as hydrodynamic diameter, zeta potential, UV-Visible spectroscopy analysis, and transmission electron microscopy. The bilirubin nanoparticles had hydrodynamic diameter of 100-150 nm, negative surface charge and spherical morphology. The synthesized nanoparticles were quite stable as observed in UV-visible spectroscopy. This nanoformulation was tested at three different concentrations i.e. 0.03%, 0.1% and 0.3% for safety evaluation upon topical application in wounded rats in which it was applied twice daily for 21 days. Evaluation for safety profile was carried out in terms of haematological and biochemical parameters and necropsy findings at 21 days post-wounding. The haematology and biochemical parameters showed no significant difference in comparison to control group. In case of necropsy, the vital organs such as liver, kidney, heart, spleen, lungs etc., showed no pathological evidence. Therefore, the synthesized nanoparticles had no systemic effects upon topical application.

Keywords: Bilirubin nanoparticles, pluronic F-127, Wistar rats, hydrodynamic diameter, zeta potential, UV-visible spectroscopy, transmission electron microscopy, safety evaluation

Introduction
An organ or tissue protrudes through an opening, which can be created by a tear in the Skin is the outer covering of the body, which acts as a protective barrier against any harmful environmental factors like pathogenic organisms, UV radiation and toxic chemicals. Apart from having a protective role, it helps regulate temperature, controls fluid losses, sensory perception, and maintains homeostasis (Lopez-Ojeda *et al.*, 2019) [13]. Any injury or insult resulting in the break within the skin ends up in wound (Boakye *et al*., 2018) [3].

The process of wound healing has been seen and documented as a natural physiological event. Integration of complex physiological events in a harmonized manner is required for optimum regeneration and reconstitution of a cutaneous wound. In the last few decades, the discovery of cellular and molecular mechanisms, and the advent of molecular biology techniques have added a lot of knowledge to the current understanding of healing mechanisms. The optimum healing mechanism occurs in a series of overlapping phases *viz*. coagulation, inflammation, migration/proliferation and tissue remodeling (Qing, 2017) [17].

Presently, about $25 billion in healthcare cost is incurred for around 6.5 million chronic wounded patients in every year (Delli Santi and Borgognone, 2019) [5]. In the United States alone, no healing wounds account for approximately $50 billion, scars from surgical incisions and trauma account for nearly $12 billion, and burns account for $7.5 billion in healthcare costs each year (Leavitt *et al*., 2016). The prevalence of acute wounds in the Indian population is 4.5/1000 people, whereas the prevalence of chronic wounds is 10.5/1000 people (Shukla *et al*., 2005) [21]. As many as 1-2% of individuals in all populations worldwide will acquire a chronic wound during their life-time (Garcia-Orue *et al*., 2017) [7]. The incidence of non-healing wounds is further increased by ageing and the increasing number of diabetic patients. Injury-related inconveniences, especially for chronic wounds, are primarily due to treatment and maintenance practices that limit wound recovery rather than tissue integrity restoration (Borena *et al*., 2015) [4].

Therefore, development of novel drugs or formulations to speed up the wound healing process is one of the frontline demands of the current era.

Recently, bilirubin (end product of heme metabolism) has shown wound healing (Ahanger *et al*., 2016) [1] as well as anti-inflammatory potential (Horsfall *et al*., 2014) [8]. It has been observed that bilirubin prevents oxidant-mediated cell death (McGeary *et al*., 2003 [15]; Kirkby and Adin, 2006) [11] and it suppresses inflammation by up-regulating protective gene heme oxygenase-1 (HO-1), (Wang *et al*., 2006) [24]. Bilirubin has also shown wound healing properties in non-diabetic and diabetic rats (Ram *et al*., 2016) [18]. The antioxidant, anti-inflammatory, anti-apoptotic and cytoprotective properties of bilirubin are ideal to act as a wound healing agent. However, poor water solubility and tissue absorption of bilirubin limit its full exploitation of different *in vivo* pharmacological actions (Watchko and Tiribelli, 2013 [25]; Yao *et al*., 2020) [27]. In order to overcome these limitations and to increase the effectiveness of bilirubin, bilirubin nanoparticles were synthesized and its wound healing potential was evaluated in Wistar rats (Kamothi *et al*., 2022) [10]. The present article covers the synthesis and characterization of bilirubin nanoparticles and study regarding the safety evaluation upon topical application and to understand the systemic effects, if any, of the bilirubin nanoparticles and bulk bilirubin, taking into account different parameters.

Materials and Methods

Reagents and Chemicals

Bilirubin (Cat. no. B4126) and pluronic F-127 (PF-127) (Cat. no. P2443) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Dimethyl sulfoxide (DMSO) of analytical grade, was purchased from Sisco Research Laboratories Pvt. Ltd. (SRL), India. Eudragit RS 100 (Ammonio methacrylate copolymer type b) was gifted from Evonik industries (Cat. no. B160208520). Mili-Q grade water was used for the preparation of all solutions.

Synthesis of bilirubin nanoparticles

Bilirubin nanoparticles were synthesized by first dissolving bilirubin (80mg) in DMSO (7.5ml) containing eudragit RS100 and kept under magnetic stirring for 20 minutes. Thereafter, Pluronic F-127 (1%) solution was prepared by dissolving 175mg of pluronic F-127 in 17.5ml of Mili-Q grade water. The solution of bilirubin and eudragit was added drop wise into the pluronic F-127 under magnetic stirring and at temperature around 20 °C for critical micelle formation occurred leading to self-assembly of pluronic micelles and the suspension was formed which was kept on stirring for 2 hours. Later, the suspension was kept under ultrasonication using probe ultrasonicator (MRC labs, Germany) at 75% intensity for 5 min at 4°C. This method was used to prepare three different concentrations of bilirubin nanoparticles i.e. 0.03% bilirubin nanoparticles [BNP (0.03%)], 0.1% bilirubin nanoparticles [BNP (0.1%)] and 0.3% bilirubin nanoparticles [BNP (0.3%)]. The ratio of 1.0:0.5 for bilirubin to eudragit was kept for the synthesis of bilirubin nanoparticles. Blank nanoparticles (Blank NP) of PF-127 were synthesized in similar manner without adding bilirubin. Brief schematic presentation of the procedure for the synthesis of bilirubin loaded PF-127 nanoparticles is shown in Fig 1. Freshly prepared different formulations were also kept undisturbed for 4 days at room temperature (22-24 °C) and their photographs were captured at different time intervals i.e. 0, 1, 3, 6, 12, 24, 48, 96 h.

Characterization of bilirubin nanoparticles

Determination of hydrodynamic diameter

The hydrodynamic diameter of bilirubin nanoparticles was evaluated using zetasizer (Malvern, UK), as per the instructions of the instrument.

Determination of maximum wavelength of bilirubin and UV-visible spectra of bilirubin nanoparticles

To determine the maximum wavelength of bilirubin, different dilutions ranging from 1-10 µg/ml in DMSO were prepared and full spectral scan i.e. λ (200nm) to λ (800nm) was carried out. Additionally, UV-visible spectral scan of bilirubin nanoparticles was also carried out at the day of synthesis and 60 days after the synthesis, along with that of the different reagents used for the synthesis of bilirubin nanoparticles. The full UV-visible spectra of bilirubin nanoparticles were done to evaluate the hypsochromic or bathochromic shift in the bilirubin nanoparticles which indicates the stability of nanoparticles.

Zeta potential of nanoparticles

Zeta potential measurements were done to determine the charge of the synthesized bilirubin nanoparticles. It was determined by Zetasizer (Malvern, UK), as per the instructions of the instrument.

Transmission electron microscopy

TEM studies of nanoparticles was done to confirm the size of nanoparticles and study the better resolution of morphology of the nanoparticles.

Procurement of Rats

Thirty-six adult healthy male Wistar rats weighing 100-140 g were procured from Disease free animal house, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana. Animal were kept in polypropylene cages and provided with free access to feed and water. An acclimatization period of a minimum of three weeks was given to the animals until the rats attained adult body weight of 150-200g before the commencement of experiment. The studies were approved by the Institutional Animal Ethics Committee (IAEC) of Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India (In its 15th IAEC meeting, agenda number 10, vide letter no. VCC/IAEC/2161-2179 dated 26-09-2019). Animals were given humane care in accordance with the National Institutes of Health Guide for the care and use of Laboratory animals (NIH Publication No. 85-23, revised 1996).

Experimentation protocol

Thirty-six adult male Wistar rats were divided into different groups with 6 rats in each group for the wound healing study. Different formulations i.e. pluronic F-127 (PF-127), blank nanoparticles (blank NP), 0.03% bilirubin nanoparticles [BNP (0.03%)], 0.1% bilirubin nanoparticles [BNP (0.1%)] and 0.3% bilirubin nanoparticles [BNP (0.3%)] were applied topically twice for 21 days at the wound site created on the thoraco-lumbar area of the rats after inducing general anaesthesia by an intraperitoneal (i.p.) injection of a combination of ketamine (50 mg/kg, i.p.) + xylazine (5 mg/kg, i.p.). At 21 days post-wounding the blood was

collected from all the rats from each group for the haematological and biochemical study. The rats were euthanized by an overdose of thiopentone (i.p.) for gross evaluation of different organs.

Safety evaluations of topical formulations

Evaluation of different haematological and biochemical parameters

After 21 days treatment of wounds with different topical formulations, blood from rats was collected in EDTA vials for analysis of different haematological parameters by using automated haematoanalyzer (Blood cell counting machine, MS4Se). Some portion of blood was also centrifuged at 700 g for 10 min to separate plasma from whole blood. The plasma sample were processed in EM 200 automated clinical chemistry analyzer for the estimation of different biochemical parameters i.e. serum glutamic pyruvic transaminase (SGPT), serum glutamic-oxaloacetic transaminase (SGOT), lactate dehydrogenase (LDH), urea, phosphorous, creatinine and protein by using commercial kits (Erba Mannheim, Transasia company).

Gross evaluation of different organs for pathological changes

Gross evaluation of different vital organs such as liver, kidney, heart, lung, spleen, etc. was done to evaluate the systemic effects of bilirubin nanoparticles.

Statistical Analysis

Results are expressed as mean ± SEM with n equal to the number of replicates. The statistical significance between the different groups was analyzed by applying one-way analysis of variance (ANOVA) as per the suitability for analysis and followed by an appropriate post-test (Tukey's or Dunnet's) using the GraphPad Prism v8.0.2 software program (San Diego, CA, USA). The differences between the different treatment groups were considered statistically significant at $P \leq 0.05$.

Results

Synthesis and Gross appearance of the bilirubin Nano suspension

The schematic representation of the synthesis of bilirubin nanoparticles is shown in Fig. 1. The representative images of the gross appearance of different formulations i.e. pluronic F-127 (PF-127), blank nanoparticles (blank NP), 0.3% bulk bilirubin (bulk B), and three different concentrations of bilirubin nanoparticles (0.03, 0.1 and 0.3%) are shown in Fig. 2. After 6 h of their synthesis, agglomeration of bilirubin particles in 0.3% bulk bilirubin formulation was observed. However, the bilirubin nanoparticles were grossly stable throughout the observation period i.e. 4 days. From 24 h onwards, there was a change in the colour of 0.3% bilirubin and three different concentrations of bilirubin nanoparticles (0.03%, 0.1% and 0.3%).

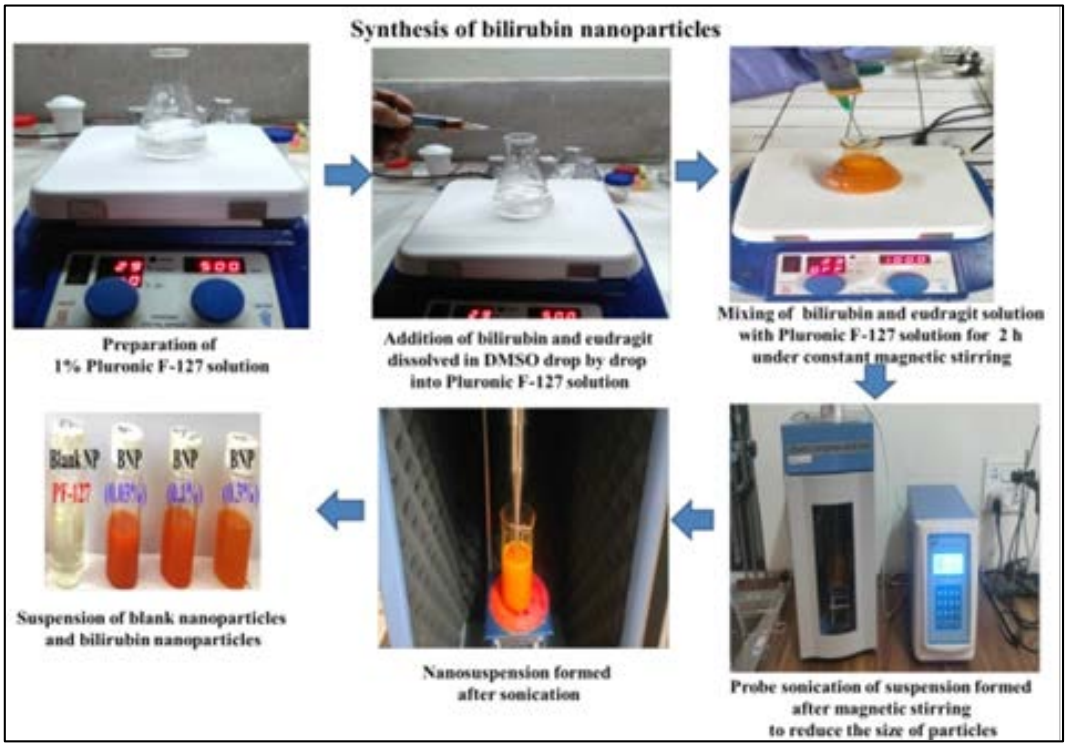


Fig 1: Brief schematic presentation of the procedure for the synthesis of bilirubin loaded PF-127 nanoparticles

Characterization of bilirubin nanoparticles

Hydrodynamic diameter and Polydispersity index (PDI) of bilirubin nanoparticles

The hydrodynamic diameter of bilirubin loaded nanoparticles in three different concentrations i.e. 0.03%, 0.1% and 0.3% was 110.85±5.79 nm, 121.63±4.26 nm and 140.38±3.44 nm (n=3), respectively (Fig. 3). The PDI of the bilirubin loaded nanoparticles in three different concentrations i.e. 0.03%, 0.1% and 0.3% was 0.21±0.02, 0.28±0.03 and 0.31±0.02 (n=3), respectively.

Maximal absorbance wavelength (λmax) of bilirubin and UV-visible spectra of bilirubin nanoparticles

Full spectral scan of bilirubin revealed that the maximal absorbance of bilirubin was at 450 nm (Fig. 4). The maximal absorbance of bilirubin nanoparticles was observed at 460 nm. The bilirubin nanoparticles showed maximal absorbance at 498 nm after 60 days of synthesis. There was slight right shift of the peak of bilirubin nanoparticles in comparison to bulk bilirubin and this right shift was more pronounced in the 60 days old bilirubin nanoparticles.

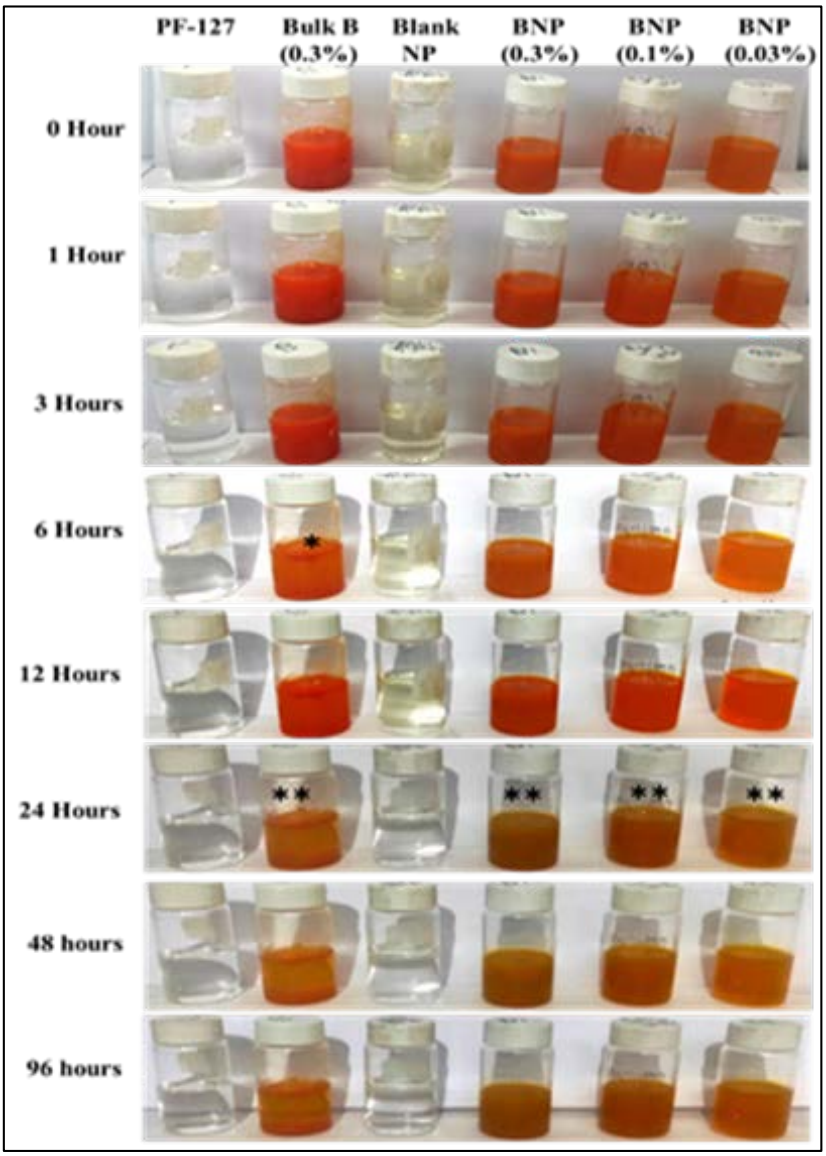
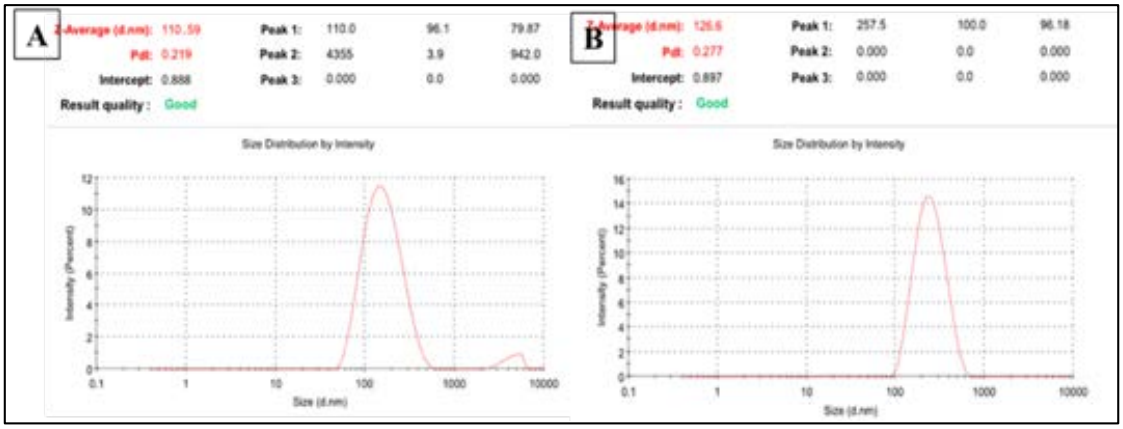


Fig 2: Photographs of different formulations at different time intervals.

These formulations i.e. pluronic F-127 (PF-127), 0.3% bulk bilirubin [Bulk B (0.3%)], pluronic F-127 nanoparticles (Blank NPs), 0.03% bilirubin nanoparticles [BNP (0.03%)], 0.1% bilirubin nanoparticles [BNP (0.1%)] and 0.3% bilirubin nanoparticles [BNP (0.3%)] were prepared for wound healing studies. ***** represents aggregation of bilirubin in bulk B (0.3%) formulation. ****** represents change in color of bulk B (0.3%), BNP (0.03%), BNP (0.1%) and BNP (0.3%) from 24 h onwards.

Zeta potential

Graphs of the zeta potential of nanoparticles obtained from Zetasizer are shown in Fig. 5. The zeta potential of blank nanoparticles, 0.03% bilirubin nanoparticle, 0.1% bilirubin nanoparticles and 0.3% bilirubin nanoparticles were -12.52±0.21 mV, -15.92±0.71 mV and -17.23±0.10 mV (n=3), respectively.



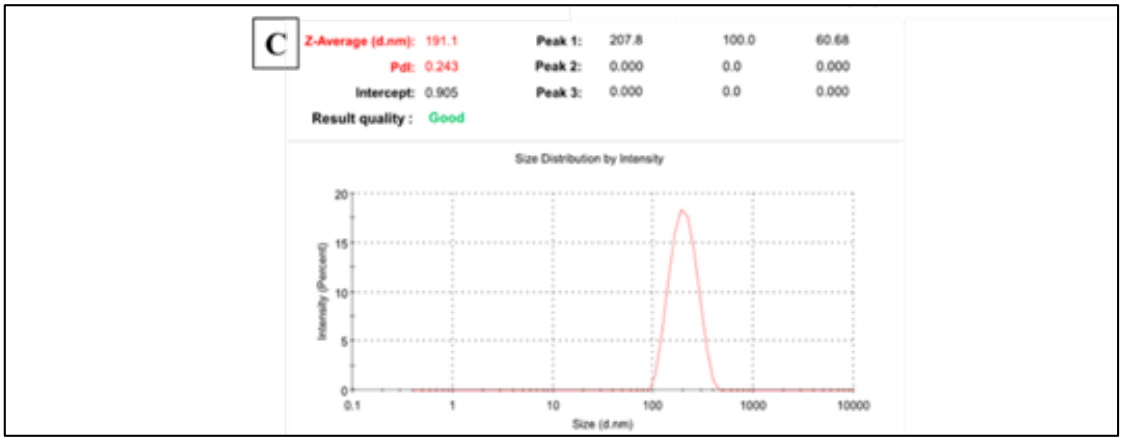


Fig 3: Graphs showing the hydrodynamic diameter (nm) and polydispersity index of (A) 0.03% bilirubin nanoparticles, (B) 0.1% bilirubin nanoparticles and (C) 0.3% bilirubin nanoparticles

TEM of nanoparticles

TEM of the bilirubin nanoparticles revealed the synthesized nanoparticles have spherical shape (Fig. 6). The size of the nanoparticles is in agreement with that of the hydrodynamic diameter of the nanoparticles obtained in the zeta sizer.

Safety evaluation of topical formulations:

Effect of bilirubin nanoparticles and other topical formulations on haematological parameters

The values of different haematological parameters after 21 days exposure of wound with different topical formulations i.e. pluronic F-127, blank nanoparticles, bulk bilirubin and bilirubin nanoparticles (0.03%, 0.1% and 0.3%) are given in Fig. 7(A-B).

The values of different haematological parameters did not differ significantly among the different treatment groups as compared to the control group. The bilirubin nanoparticles treated group did not exhibit any systemic effects as observed from the haematological values as there was no significant difference observed in comparison to the control group.

Effect of bilirubin nanoparticles and other topical formulations on biochemical parameters

The values of different biochemical parameters after 21 days exposure of wound with different topical formulations i.e. pluronic F-127, blank nanoparticles, bulk bilirubin and bilirubin nanoparticles (0.03%, 0.1% and 0.3%) are given in Table 40 and Fig. 7(C-D). The values of different biochemical parameters did not differ significantly among the groups treated with different topical formulations. In case of the bilirubin nanoparticles treated groups, there was no significant difference found among the values of SGPT, SGOT, LDH, urea, phosphorous, creatinine, and protein levels as compared to the control group.

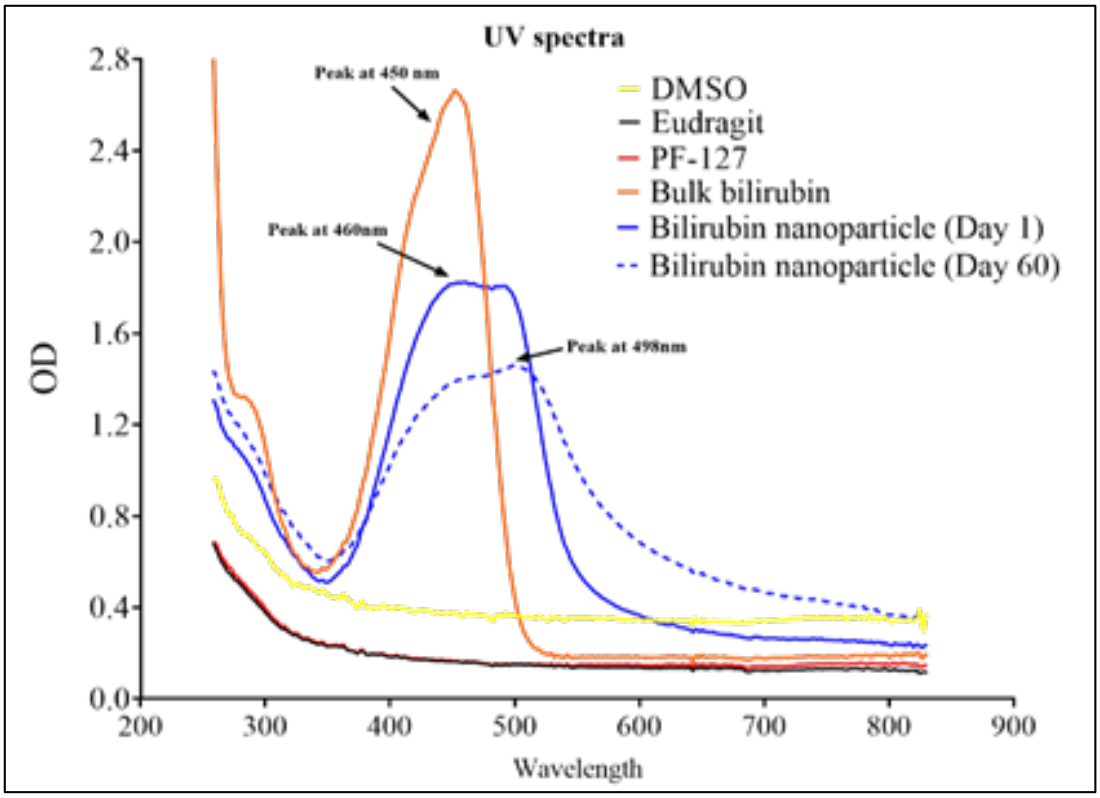


Fig 4: (A) Full spectral scan of bilirubin. (B) UV-visible spectra of DMSO, eudragit, pluronic F-127, bulk bilirubin, freshly synthesized bilirubin nanoparticles (Day 1) and bilirubin nanoparticles after 60 days of their synthesis

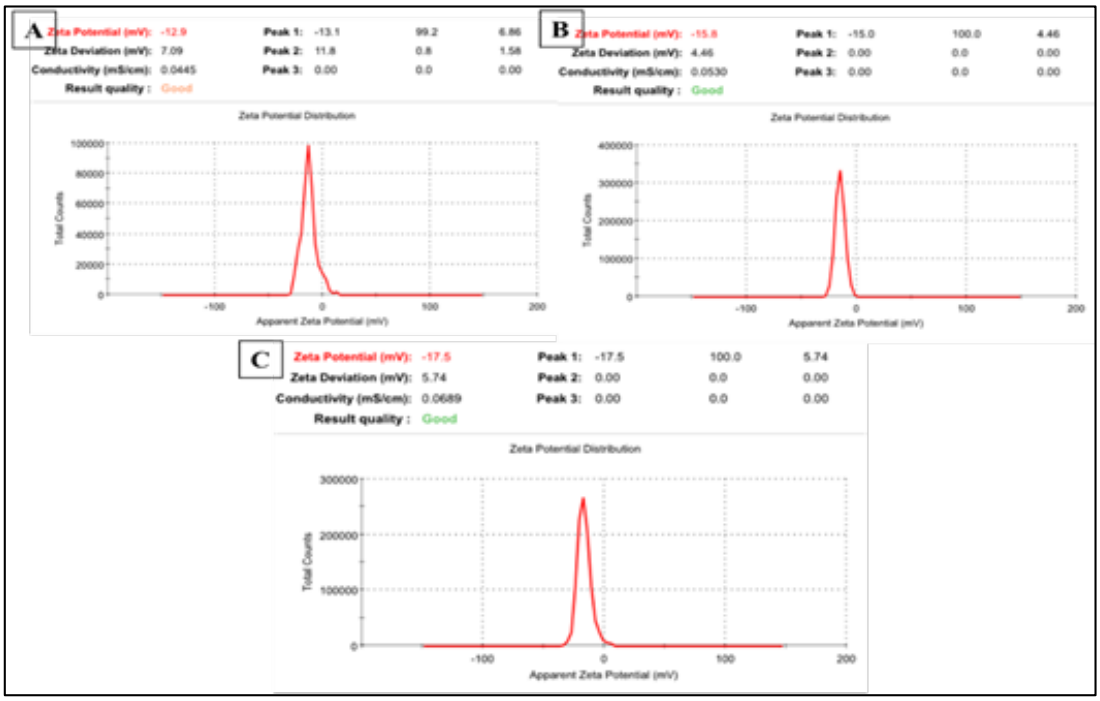


Fig 5: Graphs showing the zeta potential of nanoparticles. (A) 0.03% bilirubin nanoparticles, (B) 0.1% bilirubin nanoparticles and (C) 0.3% bilirubin nanoparticles

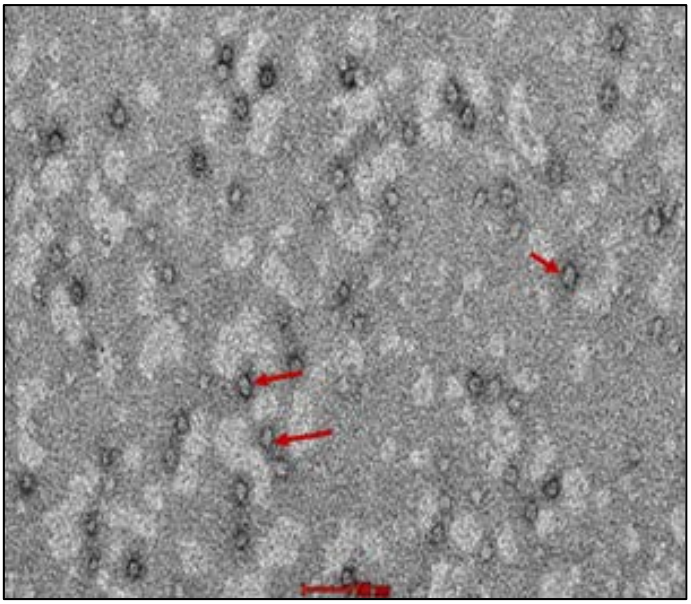
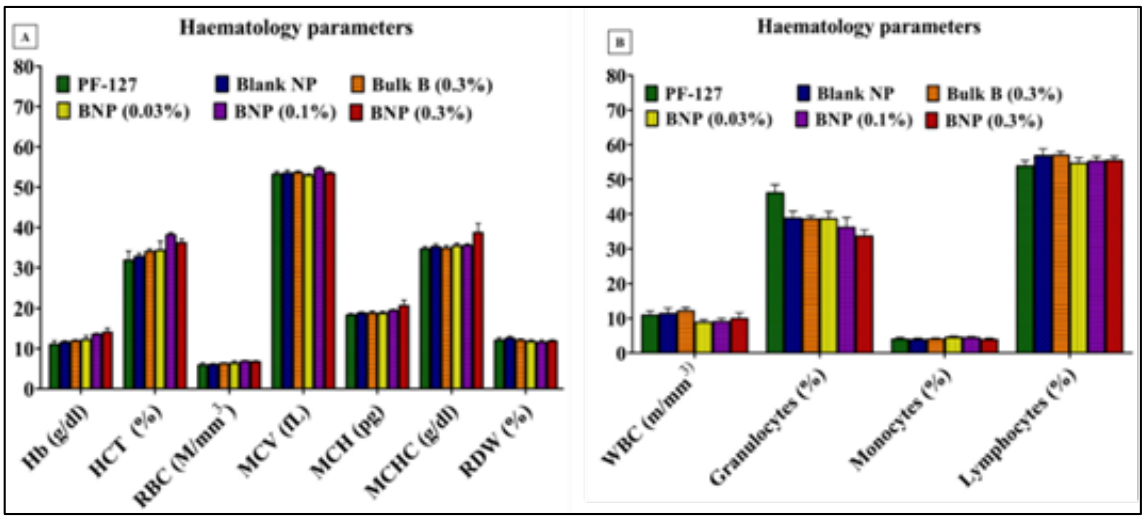


Fig 6: Transmission electron microscopy of the bilirubin nanoparticles (Arrow indicates spherical shaped bilirubin nanoparticles)



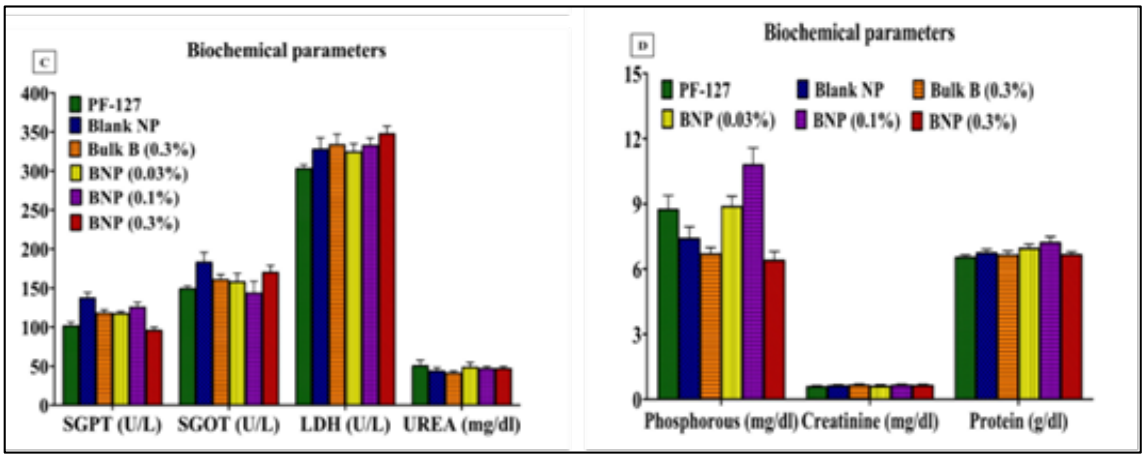


Fig 7: Effect of 21 days exposure of wounds with different topical formulations on various (A & B) haematological and (C & D) biochemical parameters. PF-127: Pluronic F-127; Blank NP: Blank nanoparticles; Bulk B (0.3%): 0.3% bulk bilirubin; BNP (0.03%): 0.03% bilirubin nanoparticles; BNP (0.1%): 0.1% bilirubin nanoparticles and BNP (0.3%): 0.3% bilirubin nanoparticles. Data are expressed as mean ± SEM (N=6)

Necropsy findings

The representative gross photographs of necropsy of wounded rats of different groups treated with various topical formulations [i.e. pluronic F-127, blank nanoparticles, bulk bilirubin and bilirubin nanoparticles (0.03%, 0.1% and 0.3%)] for 21 days post-wounding is presented in Fig. 8. The rats in different groups did not reveal any gross pathological changes in the vital organs. The bilirubin nanoparticles treated group did not show any sign of systemic effect in necropsy findings. There was no sign of change in the morphology of different organs, texture, or presence of vascular changes, swelling, exudates in vital organs such as liver, heart, kidneys, lungs, etc.

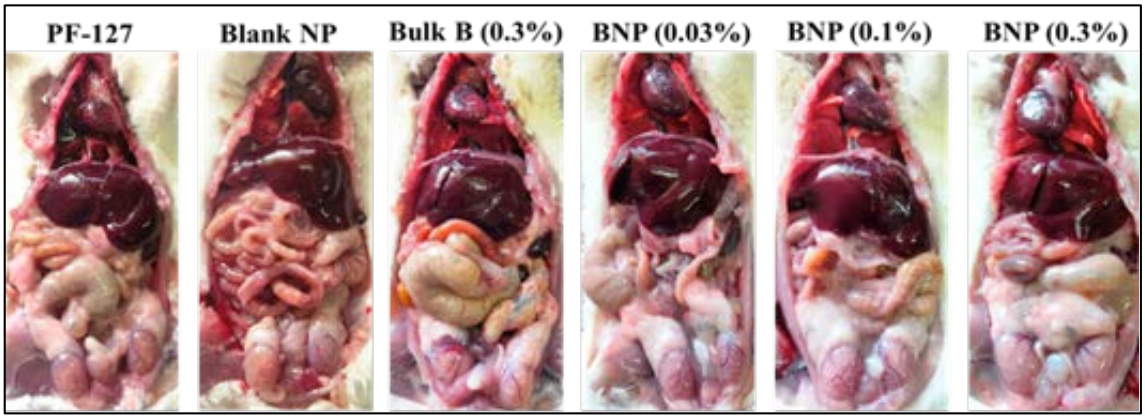


Fig 8: Representative gross photographs of necropsy of wounded rats of different groups treated with various topical formulations for 21 days post-wounding. PF-127: Pluronic F-127; Blank NP: Blank nanoparticles; Bulk B (0.3%): 0.3% bulk bilirubin; BNP (0.03%): 0.03% bilirubin nanoparticles; BNP (0.1%): 0.1% bilirubin nanoparticles and BNP (0.3%): 0.3% bilirubin nanoparticle

Discussion

Pluronics are tri-block copolymers consisting of repeating units of polyethylene oxide (PEO) and polypropylene oxide (PPO). Poly (propylene oxide) (PPO) groups make up the core block of the amphiphilic nonionic triblock copolymer pluronic, while poly (ethylene oxide) (PEO) groups make up the outer blocks (Schmolka *et al*., 1972) [19]. The ability of pluronics to self-assemble is used to produce many nanostructures (Mai and Eisenberg, 2012) [14].

Pluronic F-127, due to its excellent stability, bioadhesive qualities, thermoreversible gelling ability at room temperature, and nontoxic characteristics, makes it widely studied among the numerous pluronic species (Dumortier *et al*., 2006) [6]. The central PPO block becomes hydrophobic on exposure to high temperatures, while the PEO blocks remain hydrophilic (Israelachvili, 1997) [9]. The drug molecules accumulate in the hydrophobic PPO cores, when the hydrophobic drug molecules are mixed with suitable quantities of pluronic molecules and the temperature is raised. The hydrophilic PEO coronas prevent the drug molecules from being removed from the core and are non-toxic. Thus, the solubility of the hydrophobic drugs in an aqueous medium increase substantially, which enhances the bioavailability of the drugs (Torchilin, 2001) [23].

In the present study, pluronic F-127 at 1% w/v was used to encapsulate bilirubin in order to prepare bilirubin nanoparticles. The synthesized nanoparticles had a spherical shape, negative zeta potential and hydrodynamic diameter observed in zeta sizer were in agreement with the TEM studies. The size, shape, and core composition affect the cellular uptake of the nanoparticles (Albanese *et al*., 2012) [2]. The spherical nanoparticles have been noticed to have maximum cellular uptake (Niikura *et al*., 2013) [16]. In the present study, the gross appearance of the suspensions of bilirubin nanoparticles showed more stability with respect to bulk bilirubin in terms of particle aggregation and settlement. The color of bulk bilirubin and bilirubin nanoparticle preparations changed after their 24 h incubation at room temperature in transparent vials, which revealed their sensitivity to light. An earlier report has also revealed the

photo-sensitive nature of bilirubin (Sofronescu *et al*., 2012) [22].

In UV-visible spectroscopical analysis, the peak of bilirubin nanoparticles also falls near the peak of bilirubin, which further confirmed the presence of bilirubin in the bilirubin nanosuspension. The bilirubin nanoparticles showed peaks at 460 nm on the day of synthesis and 498 nm on 60 days of synthesis. The slight bathochromic shift observed on day 60 of synthesis indicates little aggregation of nanoparticles resulting in a right shift in UV-visible spectroscopy.

The zeta potential of nanoparticles indicates the stability and how nanoparticles interact with the cells. It has been observed that the negatively charged particles are adsorbed at the positively charged site on the cell surface through electrostatic interactions, which in turn leads to localized neutralization and further bending of the membrane to favor endocytosis of nanoparticles for cellular uptake (Win and Feng, 2005) [26]. In the present study, zeta potential revealed a low negative charge of blank and bilirubin nanoparticles. In earlier studies, thymoquinone encapsulated pluronic F-127 have also shown low negative zeta potential values, which were influenced by the nature of non-ionic surfactants pluronic F-127 (Shaarani *et al*., 2017) [20]. This might explain the negative zeta potential of the blank pluronic F-127 nanoparticles and bilirubin nanoparticles in the present study.

The safety profiles of drug delivery systems and therapeutic applications using nanoscale materials and formulations are of considerable concern. In the present study, the safety evaluation of bilirubin Nano formulation was carried out, where the bilirubin Nano formulation was administered topically in wounded rats for 21 days. To evaluate whether the topical application of bilirubin nanoparticles and other formulations has any systemic effects, the blood was collected from rats for studying the haematological and biochemical parameters. The haematological and biochemical values of bilirubin nanoparticles were in the normal range and no significant difference was observed as compared to the control group (PF-127). Additionally, no significant abnormality in all internal organs including lungs, heart, esophagus, trachea, stomach, small intestine, large intestine, liver, spleen, kidneys and testes was observed implying that bilirubin nanoparticles are non-toxic. This suggested that no systemic toxicity is associated with bilirubin nanoparticles, which can be considered safe for topical use.

Conclusion

The bilirubin nanoparticles were synthesized by our novel method using pluronic F-127. The synthesized bilirubin nanoparticles had spherical morphology, negative charge, and were stable as observed in UV-visible spectroscopy. Safety evaluation of bilirubin nanoparticles was carried out upon its topical application in wounded rats by studying haematological and biochemical profiles and observing different vital organs in necropsy findings. The study revealed that the bilirubin nanoparticles did not exhibit any systemic effects in rats upon topical use upto 0.3% concentration.

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Authors' contributions

DJK VK and VK designed the experiment. DJK, VGJ, M.A. & VK did the synthesis and characterization studies of nanoparticles. D.J.K and V.K. did the experimentation. D.J.K. & M.S. did the haematological and biochemical analysis. All authors analysed the data and contributed in writing the manuscript. V.K. supervised the whole study.

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Conflict of interest

All the authors declare no conflict of interest.

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