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In situ functionalized nanobiocomposites dressings of bamboo cellulose nanocrystals and silver nanoparticles for accelerated wound healing

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ABSTRACT

An innovative approach was adopted where *in situ* synthesized silver nanoparticles (AgNPs) from leaf extract mediated reduction of $AgNO_3$ were simultaneously impregnated into the matrix of cellulose nanocrystals (CNCs) isolated from *Dendrocalamus hamiltonii* and *Bambusa bambos* leaves, for formation of nanobiocomposites (NCs) in film and ointment forms. Here, use of plant CNCs was chosen as an alternate to bacterial cellulose for wound dressings. NCs possessing water absorption capacity and strong antibacterial activity showed synergistic effect on *in vivo* skin wound healing and documented faster and significant wound closure in treated mice. NCs exhibited lesser inflammation and early vasculogenesis at day 3 coupled with increased fibroblasts and collagen content at day 8 leading to faster neo-epithelization by day 14. Highly effective, biocompatible, and easy to apply NCs wound dressings (ointment and films) containing low amounts of Ag (0.05 ± 0.01 wt%) are potential candidates for effective skin repair.
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1. Introduction

Among biomedical applications, wound repair has been a realm of extensive research over a past few decades. The dynamic and convoluted process of wound healing engrosses the joint actions of various cell and tissue lineages undergoing inflammation, cell proliferation, migration, differentiation, and tissue remodeling events (Escamez et al., 2004). In clinical practices, effective wound healing craves for a dressing material that can cater moist environment to wound, endorse gaseous diffusion, prevents microbial infection, removes excess of exudates, and can be readily removed from the wound site without causing much pain (Jhong et al., 2014). Wound bed provides an apt environment for microbial infections leading to delayed healing (Bowler, Duerden, & Armstrong, 2001). A wide array of wound dressing materials is available in the global market, but they muster certain inevitable drawbacks such as foul smell, low swelling nature, low porosity and poor healing capacity (Kumar, Lakshmanan, Biswas, Nair, & Jayakumar, 2012). The antimicrobial agents used so far have restricted applicability due to associated toxicity issues, and development of bacterial resistance (Ye et al., 2016; Church, Elsayed, Reid, Winston, & Lindsay, 2006). Reports are available on the use of bacterial cellulose (BC) as wound dressings (Maneerung, Tokura, & Rujiravanit, 2008; Sulaeva, Henniges, Rosenau, & Potthast, 2015). However, BC isolation is tedious as maintenance of bacterial fermentation culture for several days (~10 days) is difficult, and a costly affair (Chawla, Bajaj, Survase, & Singhal, 2009). Even bacterial strains lose their ability to synthesize cellulose during their growth in culture and fail to maintain their integrity during application. Hence, emerges a need to develop and promote a dressing material fulfilling all the principal requirements.

Our pursuit is to use plant cellulose nanocrystals (CNCs) as wound dressing material since no data is available on the applicability of plant nanocellulose for wound healing. Plant cellulose is recognized as prime cellulosic source due to its vast ampleness, cost effectiveness, and fast isolation methodology of CNCs (Saito, Nishiyama, Putaux, Vignon, & Isogai, 2006). In comparison to natural cellulose, the growing interest in nanocellulose is ascribed to its remarkable features kindled by nano-sized dimensions. CNCs

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muster a plethora of unique properties like high aspect ratio (l/d), high sustainability and tensile strength, low density, inherent water uptake capacity, good mechanical, electrical, and thermal properties, biodegradability, and biocompatibility (Habibi, Lucia, & Rojas, 2010; Siro & Plackett, 2010).

In order to develop optimal wound dressing biomaterial, the current study was aimed to prove plant CNCs as state-of-the-art wound dressings for the first time using leaves of bamboo species, *Dendrocalamus hamiltonii* and *Bambusa bambos* for CNCs isolation. Bamboo was selected for this purpose as it is one of the fastest growing plants available ubiquitously in abundance as well as an affluent source of cellulose (David, 1984). Unfortunately, hydrophilic CNCs lack antimicrobial activity to prevent microbial infection around moist wound milieu (Peng et al., 2016). Therefore, we developed nanobiocomposites (NCs) by an innovative single vessel *in situ* approach, where *Syzygium cumini* leaf extract (biological reducing agent) was used for formation of silver nanoparticles (AgNPs) and their simultaneous impregnation into CNCs matrix. NCs in ointment and film forms were tested for *in vitro* antimicrobial and *in vivo* topical wound healing traits. We hypothesize that plant CNCs possessing water uptake capacity would keep wounded tissue moist by controlling wound exudates, ultimately facilitating neoangiogenesis and re-epithelization, along with synergistic effect of antibacterial and Anti-dinflammatory AgNPs accelerating tissue repair.

2. Experimental

2.1. Isolation of cellulose nanocrystals (CNCs)

CNCs were isolated from the leaves of two bamboo species, *Dendrocalamus hamiltonii* (DH) and *Bambusa bambos* (BB) by a combination of chemical (bleaching, alkali and acid treatment) and mechanical (ultrasonication) methodology as provided in detail in supporting information. Cellulose fibers obtained after bleaching and alkali treatments were deemed as chemically treated fibers (CTFs), and abbreviated for both species as CTF-DH and CTF-BB. Cellulosic structures formed after acid hydrolysis and mechanical treatments were referred as cellulose nanocrystals (CNCs) specifically as DH-CNC and BB-CNCs.

2.2. In situ *synthesis of nanobiocomposites (NCs)*

The leaf extract (LE) of *Syzygium cumini* used for synthesis of NCs was prepared using our published method (Kumar, Yadav, & Yadav, 2010). An *in situ* single vessel approach was adopted for the formation and simultaneous impregnation of silver nanoparticles (AgNPs) on CNCs matrix to develop NCs. Briefly, 150 mg of CNCs (1 wt%) were suspended in 15 mL of $AgNO_3$ solution (1 mM) and sonicated for 2 min. Further, *S. cumini* LE (10% v/v) was added and the reaction mixture was stirred for 6 h. After centrifugation, pellets containing AgNPs impregnated on CNCs matrix were regarded as NCs. To prepare NCs ointment, wet mass of NCs pellet was mixed with Vaseline® containing petroleum jelly (inert base) in the ratio of 1:1. NCs thin film was retrieved by dissolving the pellet in water, casting in a mold and allowed to dry in hot air oven. NCs were designated as DH-CNC-Ag and BB-CNC-Ag.

2.3. Characterization of CNCs and NCs

CNCs and NCs were characterized using various techniques such as DLS zeta potential, UV–vis spectroscopy, flame atomic absorption spectrometry (AAS), scanning electron microscopy (SEM), transmission electron microscopy (TEM), fourier transform infrared (FTIR) spectroscopy, X-ray powder diffractometer (XRD), and texture analyzer for determining various structural attributes. The detailed methodologies followed for characterization is provided in supporting information. For assessment of water uptake/absorption capacity, similar wt% of both types of CNCs in water was dispersed and was spread on a flat bottomed vessel to make a dried film by drying in hot air oven at 35 °C. Then water uptake capacity of these CNCs films was determined. Before weighing, excess amount of water present at the surface was blotted. These CNCs films retain some amount of water for quite a long time even after withdrawal from water, as water is not completely removed from CNCs. The water uptake was calculated from Eq. (1) where W_o is initial weight and W_t is the weight at time t.

$$\textit{Water absorption}\ (\%) = \left(\frac{W_t - W_0}{W_0}\right) \times 100 \quad (1)$$

2.4. Evaluation of bactericidal activity of NCs

The antibacterial activity of NCs (ointments and films) was tested by well and disc diffusion method, respectively, against pure strains of few gram negative bacteria e.g. *Citrobacter freundii* (MTCC No. 8128), *Escherichia coli* BL-21, *Pseudomonas aeruginosa* (MTCC No. 741), and gram positive bacteria e.g. *Staphylococcus epidermidis* (MTCC No. 435), *Bacillus subtilis* (MTCC No. 121), *Micrococcus luteus* (MTCC No. 4821) particularly responsible for wound infections and is detailed in supporting information. CNCs and streptomycin (100 μg/mL) were taken as negative and positive controls, respectively. The diameter of bacterial growth zone of inhibition (ZOI) was measured after overnight incubation. ZOI represents the area around the disc/well of antibacterial material that contains no bacterial growth.

2.5. Mechanistic study of antibacterial action of NCs

Antibacterial mechanistic action was studied by determining a change in Morphology bacteria (10^8 CFU/mL) and protein leaky content from bacteria. For this, a gram positive (*S. epidermidis*) and a gram negative bacterium (*C. freundii*) were incubated with NCs (50 mg/mL wet weight) at 37 °C for specific time intervals (0, 1, 2, 3, and 6 h) and then centrifuged. A drop of bacterial suspension from the pellet was observed for alterations in their shape under TEM. The protein leaky content in the supernatant obtained at 0 and 6 h of incubated bacterial cells was determined by Bradford assay. The absorbance of bacterial cell supernatant was taken at 595 nm, and compared with standard curve prepared from bovine serum albumin (0–10 μg/mL) to calculate protein leaky content.

2.6. In vitro *cytotoxicity studies of NCs*

In vitro cytotoxicity of NCs, DH-CNC-Ag and BB-CNC-Ag was evaluated using sulforhodamine B (SRB) assay against mice keratinocytes. The keratinocytes were isolated from 2-day old swiss albino mice following the procedure explained earlier (Lichti, Anders, & Yuspa, 2008). Experimental procedures were conducted as per protocol approved by Institutional Animal Ethics Committee (IAEC approval no. IHBTP3/Mar2015) and CPCSEA guidelines. The detailed methodology of SRB assay is given in supporting information.

2.7. In vivo *wound healing experiments*

Male, swiss albino mice, 6–8 weeks old (27–35 g) were procured from our institutional in-house animal facility (CPCSEA Registration no. 1381/ac/10/CPCSEA, DT: 27/10/2010). Experimental procedures were conducted as per protocol approved by Institutional Animal

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