Novel temperature responsive films impregnated with silver nano particles (Ag-NPs) as potential dressings for wounds

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Abstract

Silver nanoparticles have attracted wide interest in medicine on account of their antibacterial activity. We report in this paper, the antibacterial activity and biocompatibility of a temperature responsive topical film fabricated from pullulan-g-pNIPAM and impregnated with two different concentrations (15 ppm and 30 ppm) of silver nanoparticles (Ag-NPs). The release of silver from the film under the influence of temperature above the LCST has been studied and the in vitro release profile of the films has been compared with a marketed silver nano formulation, 'Meganano gel'. The release of silver from the films has a distinctive profile characterized by a sustained release over a period of 48 hrs, which is comparable to the marketed formulation. The films exhibit excellent swelling properties, making them ideal materials for absorption of exudates from wounds. The antibacterial activity of the films has been established at physiological temperature against gram-positive *S. aureus* and gram-negative *E. coli* and compared with the marketed formulation. A cytotoxicity evaluation on HeK293 cells has demonstrated their biocompatibility. The nanocomposite films are thus a new therapeutic device for management of non-healing wounds being constructed from temperature responsive polymers that release Ag-NPs when the temperature of the wound exudate is slightly higher than normal.

Keywords Ag-NPs, antibacterial films, temperature responsive, biocompatible polymer, wound healing

Introduction

Nanoparticles have been branded as "materials of the 21st century" and have a wide spectrum of use in drug delivery, industrial fields, biomedical, electronics, engineering and environmental applications [1-4]. In regard to drug delivery systems, nanoparticles have demonstrated benefits over conventional technologies such as increase in the solubility of hydrophobic drugs, controlled release of an encapsulated drug, targeted treatments when fabricated with cell specific ligands and the ability to impart stability to therapeutic agents by physical or chemical modifications [2]. Of the various types of nanomaterials, metallic nanoparticles have cornered significant attention because of their potent antibacterial activity via chelation or protein precipitation mechanisms [5].

Antibiotic resistance is one of the most serious health threats due to adaptation of microorganisms to the drug molecules. This has brought into focus certain metal drugs that were once used to treat various infections before the existence of current antibiotic therapies [6]. The most widely used metal is silver in the form of nanoparticles (Ag-NPs). The large surface area provided by the nanoparticles affords better contact with the microorganism cell membrane, allowing it to penetrate the microorganism. It is probable that the Ag-NPs attack the respiratory chain and prevent cell division that finally leads to bacterial cell death [7-9]. Ag-NPs ranging in size from 1-100 nm can be fabricated by chemical, physical or biological synthesis [5, 10, 11].

In silver formulations, the control of release of silver ions from a delivery system is of utmost importance for prolonged and efficient antibacterial effect. Ag-NPs have been combined with various biocompatible polymers formulated as nanocomposites like topical hydrogels and fabricated as dressings and mats for antimicrobial applications [12-15]. Kalaivani *et al*. [16] and Smiechowicz *et. al.* [17] have reported chitosan and cellulose [18] nanosilica composites loaded with Ag-NPs respectively as antibacterial agents against a range of microorganisms. Alginate, a natural polymer, has also been combined with silver as an 'alginate silver' composite for treating burns by Opasanon *et.al.*[19]. These studies suggest a wide scope for enhancement of the antibacterial properties of Ag-NPs by combining them with natural polymers.

The natural polymer pullulan is water soluble, has high adhesiveness and possesses exceptional gelling and film forming properties. Pullulan films are transparent, edible, non-toxic, thermally stable and with elastic properties making it an attractive material for packaging, food processing and various pharmaceutical applications [20, 21]. In addition, it can be chemically modified or combined with other polymers to expand its functionality and scope of application. Pullulan in the form of films has been reported by Priya *et. al.* [22] for wound healing of incision wounds. It is also used as a composite with other polymers for wound healing applications; Garg *et. al* [23]. have reported a biomimetic collagen-pullulan and carboxymethyl pullulan tyramine [24] hydrogel in wound healing of adipose-derived mesenchymal stem cells and in engineering of cartilage tissue. Pullulan-collagen hydrogel by Rustad *et. al.* [25] and Gniewosz *et.al.* [26] have shown antibacterial activity and enhancement of angiogenic capacity of mesenchymal stem cells, thus aiding the healing of wounds. Ag-NPs have also been

combined with pullulan by Kanmani *et.al.* [27] and found to have excellent antibacterial and antibiofilm activity against multidrug resistant bacterial pathogens. A pullulan derivative, 6-carboxypullulan (a reducing agent) in combination with Ag-NPs has been shown to be effective against gram-positive and gram-negative bacteria. However, pullulan has not been much explored in combination with Ag-NPs for healing of wounds. This opens avenues of modification of pullulan in combination with Ag-NPs for antibacterial application.

Temperature responsive materials have been widely explored for delivery of various nano materials as well as drugs. Below the transition temperature or the LCST (lower critical solution temperature), the drug loaded polymer systems swell in water because of hydrogen bonds between the water molecules and the functional groups of the polymers. When the temperature is increased beyond the LCST, a hydrophilic-hydrophobic transition occurs, accompanied by a coil-to-globule change in morphology. The shrinkage is accompanied by release of the loaded drug from the polymer network in a continuous manner while the temperature is maintained above the LCST. The temperature responsive nanocarriers are one of most important groups of smart nanoparticles (NPs) delivery systems that have been investigated in recent times. Dini *et al* and Nakagami *et al* have reported varied temperature ranges for different types of wounds; the exudate from a healing wound is at a lower temperature (33°C) whereas the acute wound or worsening inflamed wound has a higher temperature (35°C or more) [28, 29]. Antibacterial wound dressings as semi-interpenetrating network (semi-IPNs) composed of thermoplastic polyurethane elastomer crosslinked with poly(*N*-isopropylacrylamide) (pNIPAM) and loaded with silver nanoparticles (Ag-NPs) have been reported by Abdali *et.al.*[30] These temperature responsive antibacterial wound dressings were studied for various attributes like mechanical strength, efficiency in handling wound exudates and ease of peeling from the wounded area. The antimicrobial activity of the dressings against various gram-positive and gram-negative bacteria as well as fungal strains has also been demonstrated at the LCST.

The use of Ag-NPs as antibacterial agent has been well established. On the other hand, pullulan and temperature responsive polymers individually have been used to prepare wound dressings with antibacterial properties. But pullulan combined with a temperature responsive polymer, loaded with Ag-NPs and fabricated as a film has never been reported for wound healing. However, the graft polymeric assembly of pullulan and pNIPAM (GPA-04) has been explored by us for wastewater treatment and the outcomes have been published [31]. The unique swelling property of this composite at LCST and its inherent antimicrobial property has prompted us to explore it for wound healing purposes. The antibacterial property of Ag-NPs, the biocompatibility of pullulan, and the delayed release of the payload from the temperature responsive polymer film makes the formulation a winning combination that can be a value addition to existing therapies in wound healing management. The present study describes films made from a temperature responsive polymer grafted with the natural polymer pullulan and impregnated with silver nanoparticles (Ag-NPs) as an antibacterial medicament for wound healing.

Experimental

Materials

The natural polymer pullulan (Mol wt. 200,000 Da.) was a gift sample from Nagase India Pvt. Ltd. (Mumbai, India). The monomer N-isopropylacrylamide (NIPAM) was a gift sample from SLN Pharma Chem (Mumbai, India). The free radical initiator ceric ammonium nitrate (CAN) was purchased from S.D. Fine Chemicals (Mumbai, India) and dialysis membrane (12000-14000 Da) from Hi-media (Mumbai, India). Silver nitrate, trisodium citrate, propylene glycol and glycerol were purchased from S.D. Fine chemicals, Mumbai. For cytotoxicity studies, Dulbecco's modified eagle medium (DMEM) and foetal bovine serum (FBS) were obtained from Hi-media, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich, USA. Nutrient agar, nutrient broth and dialysis membrane (molecular weight cut off 12000-14000 Da) were also purchased from Hi-media.

Synthesis of graft polymeric assemblies (GPA) and characterization

Briefly, the GPA assembly was prepared by a free radical polymerization method using pullulan, ceric ammonium nitrate in nitric acid solution and NIPAM at 25°C under nitrogen atmosphere for 20 hrs. The detailed method of synthesis and structural elucidation of the synthesized polymer using FTIR, ^{1}H-NMR, DSC and molecular weight determination have been discussed in our previously published paper [31]. The product as the copolymer pullulan-*g*-pNIPAM (GPA-04) was fabricated as films and impregnated with Ag-NPs. The temperature of a 2.5% solution of the copolymer was linearly increased from 20 to 50°C at 1°/min and the temperature at which the solution turned turbid was noted as the LCST. Further the LCST reading was validated using a Mettler (Toledo) DSC 822e unit.

Viscosity and molecular weight:

The GPA-04 composite was tested for kinematic viscosity and shear rate with the Anton Paar Lovis 2000 M/ME (S/N 83296437) rolling ball viscometer. Viscosity was measured using the principle of time taken for a rolling ball to fall through the glass capillary containing a 0.5%w/v of GPA-04 polymer solution under gravitational force at the Lovis temperature 30°C and Lovis angle 30°.

Molecular weight of GPA-04 was determined using a Waters Alliance (Waters e2695) HPLC with RI (Waters 2412) detector. The sample was eluted through an ultra-hydrogel column using 0.1 M sodium nitrate buffer solution as the mobile phase, with a flow rate of 0.6 ml/min. Pullulan samples from 6,300 Da to 642,000 Da were used as the calibration standards for calculation of the molecular weight of the samples.

Cytotoxicity evaluation of GPA-04 [32]

The cytotoxicity studies of GPA-04 were carried out by the MTT cell viability assay. For the assay, human embryonic kidney (HEK293) cell line was replicated and used. Initially, the cells were thawed in a water bath at 37°C and centrifuged with 5.0 mL of Dulbecco's modified eagle medium (DMEM) and 10% foetal bovine serum (FBS) for 5 min at 2000 rpm to remove DMSO. The supernatant was discarded, and the cell pellets were resuspended in 1.0 mL of cell culture medium. The cells were

counted in a Neubauer chamber followed by seeding into cell culture dishes. After culturing, the cells were calculated as described and used in cytotoxicity evaluation. For MTT cell viability assay, cells were seeded into 96-well plates at 1×10^4 cells/well with 100 µL of DMEM and FBS and cultured for 24 hours at 5% CO_2 and 37°C in an incubator. After the incubation, DMEM and FBS were removed and replaced with 100 µL of DMEM and FBS and with increasing GPA-04 concentration (100, 250, 500 and 1000 µg/mL) in triplicate. After 24 and 48 hours of incubation, the respective media were removed and 100 µL of MTT solution (1 mg/mL in DMEM-FBS) was added to each well and the plates were incubated again for 4 hours at 37°C. The unreacted MTT solution was then removed and finally, 100 µL of DMSO was added to the plates to dissolve the formazan crystals, and the absorbance of the wells was recorded at 550 nm using a microplate reader (Thermo-scientific). The untreated cells were used as positive control. The experiments were done in triplicate (n = 3) and the cell viability was calculated using the following equation (1),

$$Cell\ viability = \frac{Average\ absorbance\ of\ cells\ incubated\ with\ GPA}{Average\ absorbance\ of\ cells\ incubated\ with\ DMEM + FBS} \times 100 \qquad (1)$$

Preparation of colloidal silver nanoparticles (Ag-NPs)

Capping agent trisodium citrate solution, 10 ml (1%w/v), was added dropwise at a rate of 0.15 ml/min to a boiling 100 ml silver nitrate solution (0.001M) under magnetic stirring (800 rpm). The ratio of silver nitrate solution to trisodium citrate solution was 10:1. Heating was continued at 95°C till a change in colour was observed; then the solution was gradually cooled to room temperature under continuous stirring. Optimization of various parameters was done on a trial-and-error basis.

Characterization of Ag-NPs

The particle size was initially determined using a Malvern zeta sizer and the surface morphology of the Ag-NPs was studied by scanning electron microscopy (SEM). The average size was confirmed from images captured with a TESCAN VEGA3 microscope.

Preparation of Ag/pullulan composite films

Films of GPA-04 impregnated with Ag-NPs were prepared by the casting-solvent evaporation method. Briefly, Ag-NPs colloidal solutions of variable content (15 and 30 ppm) were added to a 10% (w/v) GPA-04 aqueous solution (30 ml) and the mixture stirred for 30 minutes. To promote the plasticization, 15% of propylene glycol i.e., 0.46 ml (in respect of GPA-04) was added into the mixture. The solution was further stirred for 45 minutes; the solution was then poured into glass petri-plates of fixed diameter. These plates were dried in a desiccator for 24 hours.

Optimization of formulation variables

Effect of various formulation variables such as concentration of GPA-04, volume of GPA-04 solution, type of plasticizers and concentration of plasticizer on film characteristics was investigated. The optimization was done by evaluating the films formed with respect to the following parameters:

i. Visual appearance - the films were analyzed for their visual appearance. Any irregularity seen was noted.

ii. Thickness - the thickness of the films was measured using a digital Vernier caliper with an accuracy of 0.05mm. It was measured for each film at five different sites and the mean calculated[33].

iii. Folding endurance - it was determined by repeatedly folding manually (max 300) the film at the same place till it broke or folded. The folding endurance is the number of times the film could be folded at the same place without breaking[33].

iv. Density (g/cm^3) - Density of GPA-04 films impregnated with 30 ppm Ag-NPs was determined by equation (2). The mass of a 1 cm^2 (width 1.0 cm, length 1.0 cm) piece of film was 17 mg, and thickness 280 µm. These values were used to calculate the density according to equation 2.

$$Density = mass/(width \times length \times thickness) \quad (2)$$

The average weight of the film was from triplicates (n=3) measurements.

v. Swelling characteristics - to study the swelling index of the films, a film with area 1.0 cm^2 was weighed (w_1) and then immersed in phosphate buffer pH 7.4. It was periodically removed and weighed (w_2) after gently drying with a filter paper to remove surface liquid and the percent swelling was calculated as stated in equation. (3)

$$\text{Swelling Index} = \frac{(W_2 - W_1)}{W_1} \times 100 \quad (3)$$

vi. Silver content - silver content of the films was estimated by dissolving a film with area 1.0 cm^2 in 10 ml of phosphate buffer pH 7.4 using a magnetic stirrer. An aliquot 0.1 ml was withdrawn and diluted to 10 ml with deionized water. The silver content was quantified using a Teledyne Leeman Labs Prodigy 7 ICP-OES system.

vii. *In-vitro* release - to determine the release of silver, GPA-04 films with area 1.0 cm^2 were placed in a dialysis membrane (12000-14000 Da) and then immersed in a beaker containing 100 ml phosphate buffer pH 7.4. This setup was shaken at 37°C in a constant temperature water bath. Aliquots were withdrawn at 0, 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 hours and the aliquots were replaced by an equivalent amount of buffer. The silver content was then determined by ICP-OES.

viii. As mentioned earlier, the films made in this work were compared with the marketed gel formulation. For the marketed formulation, 1 gm of the gel was introduced into the dialysis bag and the release profile studied.

Antibacterial studies

The antimicrobial activity of the films loaded with Ag-NPs was done by the diffusion method on agar plates on selected gram-positive (*S. aureus*) and gram-negative (*E. coli*) organisms. The medium was prepared by dissolving nutrient agar in distilled water. This was then sterilized by autoclaving at a temperature of 121°C and 15 psi pressure for 15 minutes. Bacterial culture, previously grown in nutrient broth was added to the sterile nutrient agar to obtain a final bacterial concentration of 10^6 cfu/ml. This

medium was poured into sterile petri plates under a laminar air flow and allowed to set. Circular films of 1 cm diameter were placed on the agar under laminar air flow and then incubated at 37°C for 24 hours; the zone of inhibition was then measured. This was done in triplicate with each film for each organism and an average diameter of the zone of inhibition was noted. The zone of inhibition of the composite films containing 15 and 30 ppm of Ag-NPs were compared with the zone of inhibition for the marketed formulation containing 20 ppm of silver in nano form.

Results and discussion

Determination of LCST

The cloud point and differential scanning calorimeter (DSC) endotherm confirm the LCST of the synthesized polymer. The LCST of pNIPAM homopolymer is 32°C while that observed for the graft polymeric assembly (GPA-04) is 35.2°C. This shift in the LCST is because of changes in the hydrophilic properties of the material, resulting from the addition or changes in the functional groups by the graft. A similar shift in the LCST values was evident in our previous work [31] and the results were confirmed by DSC.

FT-IR and ^{1}H-NMR analysis

The structural characterisation of GPA-04 by FTIR and ^{1}H-NMR has been reported in the journal *SN Appl. Sci* [31].

Physical properties

Viscosity: Rheological properties can affect the characteristics of the polymer which is dependent on the quality of fabricated products. Viscosity is a key rheological property, and changes with increasing shear rate. The kinematic viscosity and shear rate of polymeric films were measured for pullulan and GPA-04. Viscosity is dependent upon shear stress; it is a magnitude of internal friction that provides resistance to uniform flow as measured by the force per unit area. Pullulan itself, has a higher viscosity compared to GPA-04 which is evident from the low shear rate of pullulan as compared to GPA-04 as indicated in Table 1. Reports indicate that viscosity is an important factor for topical formulation as it may influence the release of drug by altering the diffusion rate. Highly viscous polymers will greatly obstruct the drug release in contrast to low viscosity polymers [34, 35].

Molecular weight: GPA-04 has a lower molecular weight compared to pullulan; the molecular weight is 86.5 kDa. This indicates that while grafting the high molecular weight pullulan breaks up to form GPA-04 and other oligomers.

Cytotoxicity evaluation of the GPA-04

For dressings used for wound healing, biocompatibility is of prime importance. HeK293 cell lines have been extensively used to evaluate cytotoxicity of materials for wound healing applications [36]. Thus, cytotoxicity studies of GPA-04 were carried out by the MTT assay in HEK293 cell line.

For GPA-04, the percentage cell viability was calculated using Eq. (4) as mentioned earlier and the results are shown in Fig. 1; no toxic effects are observed in the control group. The cell viability values are 94%, 89% 86% and 82%; while in tests carried out for 48 hours, the values are 89%, 85%, 82% and

79% respectively. After 24 hours, the HeK293 cells are found to be viable in the acceptable range relative to the control, at GPA-04 concentrations as high as 1000 µg/mL. However, after 48 hours incubation with GPA-04, the percentage viability of the HeK293 cells decreases slightly but the difference is not statistically different from the results obtained after 24 hours [37]. Moreover, Shao W et al. have stated that samples displaying cell viability values larger than 75% are considered as non-cytotoxic and are considered excellent materials for wound dressing. The cytotoxicity of GPA-04 has been measured in this study, however for the individual components, such an exercise was not carried out, since the non-cytotoxic nature of the individual components *i.e.* pNIPAM and propylene glycol used in formation of the film has been reported in the literature [38, 39].

Characterization of Ag-NPs

The Ag-NPs were characterized by SEM and the results are shown in Fig. 2. The particle size obtained by zeta sizer was confirmed from by SEM. The average particle size obtained for Ag-NPs was found to be 55-80 nm. The SEM images show the random distribution in size and confirm the fabrication of silver nanoparticles.

Optimization of formulation variables and characterization of films

Properties of the polymeric film are dependent on parameters such as concentration & volume of polymer solution used for preparation of the film and the plasticizer used for making the film along with its concentration. Ideally, the film should be flexible, free of air bubbles and be able to modulate the release of the active component. Thus, parameters affecting the characteristics of the GPA-04 film were investigated and optimized. Also, to study the effect of the polymer on film properties, different concentrations of GPA-04 and different volumes of the same concentration were used to prepare films and the changes in the properties of the film were studied. These are summed up as follows:

i. Effect of different concentrations of GPA-04 solution

Different concentrations tested were 5%, 10%, 15% and 20%.

The films formed with 5% concentration of polymer are extremely thin and fragile. It is difficult to peel off the sample from the petri dish without breaking. Solutions of 15% and 20% GPA-04 are highly viscous and present difficulties in handling. It is difficult to pour and disperse Ag-NPs uniformly into these viscous solutions. The films formed with 10% GPA-04 have moderate thickness and are mechanically stronger and do not break while peeling. The solution is also less viscous than the 15% concentration. The thickness of the film is found to increase as the concentration of GPA-04 increases. The best result in terms of thickness and ease of removal of film from the petri dish is with 10% concentration and this was selected for further studies.

ii. Effect of different volumes of GPA-04 solution

Different volumes 10, 20, 30 and 40 ml of a 10% concentration of GPA-04 was poured into a petri dish to form the film. It is observed that the thickness of the film increases with increase in volume poured, which is due to increase in the polymer amount with respect to the fixed surface area. For wound healing, films with thickness in the range of 250 to 280 µm are considered as optimum in terms of ease

of peeling and flexibility. The films formed with 30 ml of polymer solution give the best film in terms of thickness (280 µm) and ease of removal from the petri dish. Hence, this volume of 10% GPA-04 was used for further studies. Also, it is necessary for the final film to be flexible to adhere to the wound after application. To study the effect of different plasticizers on film properties, two different plasticizers were tried, glycerol and propylene glycol. It is found that propylene glycol has better plasticizing effect on the films. Films formed with glycerol exhibit a tendency to break while peeling off from the glass petri dish. Hence, different concentrations of propylene glycol were tested and the effect on film properties was studied.

To optimize the concentration of propylene glycol, films containing 5%, 10%, 15% and 20% propylene glycol (percent with respect to dry polymer weight) were prepared. As the concentration of propylene glycol increases the hardness of the films decreases. Films with 5% and 10% propylene glycerol are considerably harder than the ones formed with 15% and 20%. Films with 20% propylene glycol are very soft and difficult to remove from the petri dish. Hence 15% propylene glycol was the final concentration used to form films, which were then tested for their antibacterial activity.

iii. Characteristics of the optimized films

The blank films are transparent, while the Ag-NPs loaded films have a yellowish-brown color which is because of the impregnated silver nanoparticles. The surface of the films appears smooth and clear without entrapment of air bubbles.

Film thickness is an important property which can influence the time required to release the silver nanoparticles on to the oozing wound. The determination of uniform thickness of each film was done with a digital vernier calliper at three different sites on the films. The optimized films are 280 µm thick. These films are easy to peel-off from the glass plates without breaking and are flexible and easy to handle.

Density increases with increasing solid content, and this reduces the flexibility of the films [40]. Density of the GPA-04 film (30 ppm Ag-NPs) was found to be 0.608 gm/cm^3, suggesting good flexibility of the films. Folding endurance above 300 indicates good mechanical strength. High folding endurance prevents easy dislocation of the film from the site of application and is less prone to breakage during use or storage. The folding endurance of the films is 310 $\pm$ 5 folds, consistent with the folding endurance range reported for polymer films used for wound healing.

Swelling of the films was found to be 110%. Percent swelling at different time points is given in Fig. 3. The films swell quickly in phosphate buffer pH 7.4, swelling to almost 95% in 0.5 hour. This is beneficial in case of a wound with exudates, as the film will quickly absorb the exudates. Reports reveal that a temperature responsive architecture helps to improve the swelling property of the overall composite material, and in the current study, the swelling property is due to pNIPAM that has been grafted on to the pullulan backbone.

Percentage silver content of the films was quantified and compared with the marketed formulation by ICP-OES. The 30 ppm Ag-NPs GPA-04 film has a silver loading of 96±1.9%. This implies that a significant amount of silver nanoparticles has been impregnated in the films. In 1.44 gm of the film, the amount of Ag-NPs has been estimated as 28.8 µg, which means every gram of the 30 ppm film contains approximately 0.02 mg of Ag-Nps. The label claim of the marketed formulation, for each gram of the gel, is 0.02mg of Ag-NPs. In past few decades, Ag-NPs have been extensively explored as an antibiotic alternative wherein the qualitative and quantitative analyses of silver ions have confirmed the concentration dependent bactericidal activity of Ag-NPs, which could also apply to the GPA-04 film [41]. Silver ions display multiple antimicrobial mechanisms against bacteria. Some of these modes of action are: blockade of the transport of the nutrients through the cell wall, collapse of the plasma membrane, attack of the respiratory cytochrome and prevention of transcription of DNA and RNA, all of these ultimately preventing cell division and death. Bacteria are less likely to develop resistance against these modes of action of silver ions[42].

In-vitro release

The films were evaluated for *in vitro* release of silver at physiological temperature. Cumulative release as shown in Table 2 was found to be 92.9% at the end of 48 hours. The release curve shows an initial burst of ions, which could be due to the composite film in solution has taken a lag time for sol to gel transition. However, when the film was stabilized at physiological temperature, the swollen matrix showed controlled release of silver ions. In case of wound healing, an initial burst will provide immediate relief, following which, a slow prolonged release will promote gradual healing as reported by Muhammet *et al* [43]. GPA-04 composed of the non-biodegradable temperature responsive pNIPAM and the biodegradable pullulan can be regarded as a matrix type device for Ag-NPs. The release the antibacterial silver ions occurs at the phase transition temperature 35°C which is close to the inflamed critical wound temperature. The pNIPAM scaffold has been reported to provide a spatial and temporal controlled release strategy for drugs. The rate of diffusion of silver ions through the matrix is driven by the degree of swelling of GPA-04, with increasing flux of silver ions in the medium at the LCST [44, 45].

The release of silver nanoparticles from GPA-04 is contrasted with the marketed formulation (Zuventus Healthcare - 0.02 mg/gm silver as nanoparticles) in Table 2. Although the marketed formulation shows a quicker release of silver ions in the early stage, the two formulations are comparable as regard the total amount of silver released. The release of Ag-NPs from the composite films lags the marketed formulation in the earlier stages, this is because matrix characteristics (gel vs film) from which the silver ions are being released are unique. Formulations of silver nanoparticles alone and silver nanoparticles embedded in pullulan films intended as an antimicrobial agent in wound healing, have displayed just a burst release of silver ions [46]. The GPA-04 films are superior in that they provide an initial burst of

silver ions for an immediate antibacterial effect; this is followed by a slow release of silver ions that offer a sustained antibacterial effect.

Antibacterial studies

The composite films were evaluated for antibacterial activity against gram-positive and a gram-negative bacteria namely *S. aureus* ATCC 6538 and *E. coli* ATCC 8739 respectively and the results are shown in Figs. 4 (a) and (b) respectively. The zone of inhibition was found to be much broader in the case of films loaded with 30 ppm Ag-NPs compared to 15 ppm. In comparison, the zone of inhibition with 1 gm (20 ppm Ag-NPs) of marketed formulation was found to be comparable with composite films loaded with 30 ppm Ag-NPs. In addition, the release data reveals that the total silver released from the films is marginally higher than the marketed formulation. Table 3 shows the zones of inhibition for the composite films vis-à-vis the marketed formulation for both bacterial strains. In addition to this fact, the films have shown excellent swelling property, which is essential for absorption of exuding or abrasive wounds. Though the physical characteristics of gels (semisolid) and films are (solid) are slightly different, both offer easy and accurate applications. Also, both gels and films are good drug reservoirs, however topical films have better patient compliance over gels [47]. The physical characteristics of propylene glycol when used as a plasticizer in making films of GPA-04 provide an environment that can shield the wound from direct contact with the external environment.

To the best of our knowledge, the composite pullulan-pNIPAM graft (GPA-04) impregnated with silver nanoparticles with a LCST of 35°C, is a first of its kind formulation, designed for wound healing.

Conclusions

Silver nanoparticles have a prominent role in the field of nanomedicine. We have prepared a composite film from pullulan and pNIPAM and impregnated with Ag-NPs that is temperature responsive, as a medication for wound healing. The release of silver nanoparticles from the composite films parallels that observed for the marketed gel. The antibacterial effect of the composite films and the marketed gel are comparable and concentration dependent. However, it is important to realize that the distinct advantage of films over gels is patient compliance in terms of easy of application and removal. Thus, temperature responsive composite films impregnated with silver nanoparticles have an excellent potential as a topical application for bacterial infections.

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Table 1: Kinematic viscosity of GPA 04 and pullulan

	Pullulan	GPA-04
Shear rate s^{-1}	353.6	427.9
Kinematic viscosity mm^2/s	1.133	0.937

Table 2. % Cumulative release of silver from GPA-04 impregnated with Ag-NPs films measured by ICP-OES

	Films	**Formulation A**
Time (hours)	**Mean ± SD**	**Mean ± SD**
0	0	0
0.5	22.4 ± 0.49	45.1 ± 0.53
1	32.2 ± 0.49	52.0 ± 1.55
2	40.2 ± 0.90	61.3 ± 1.28
4	51.1 ± 0.49	69.1 ± 1.02
6	62 ± 0.82	76.0 ± 0.98
8	72.9 ± 1.22	83.2 ± 1.47
12	80.2 ± 0.33	88.1 ± 1.72
24	89.8 ± 0.69	90.0 ± 0.90
48	92.9 ± 0.78	90.5 ± 1.65

Table 3. Zone of inhibition for GPA-04 impregnated with Ag-NPs films against bacterial strains *S. aureus and E. coli*

Bacterial strain	Sample	Zone of inhibition (mm)
S. Aureus	15 ppm composite film	30.0 ± 1.1
	30 ppm composite film	43.3 ± 2.8
	1 gm formulation A	38.3 ± 3.2
E. Coli	15 ppm composite film	32.0 ± 1.7
	30 ppm composite film	42.0 ± 1.52
	1 gm formulation A	39 ± 1

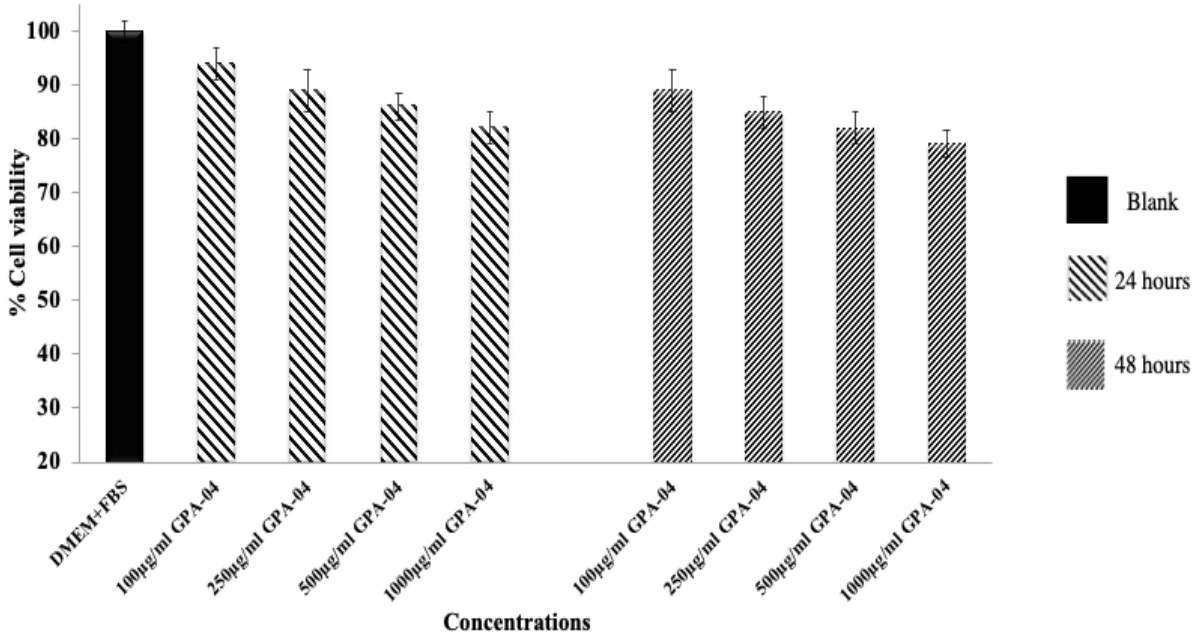

Fig. 1. Cytotoxicity of GPA-04 studied in HeK293 cells

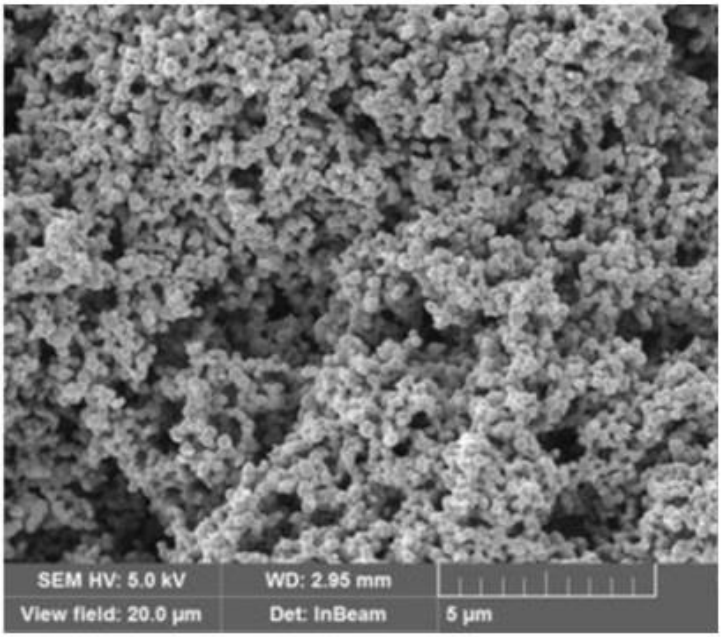

Fig. 2. SEM image of Ag-NPs

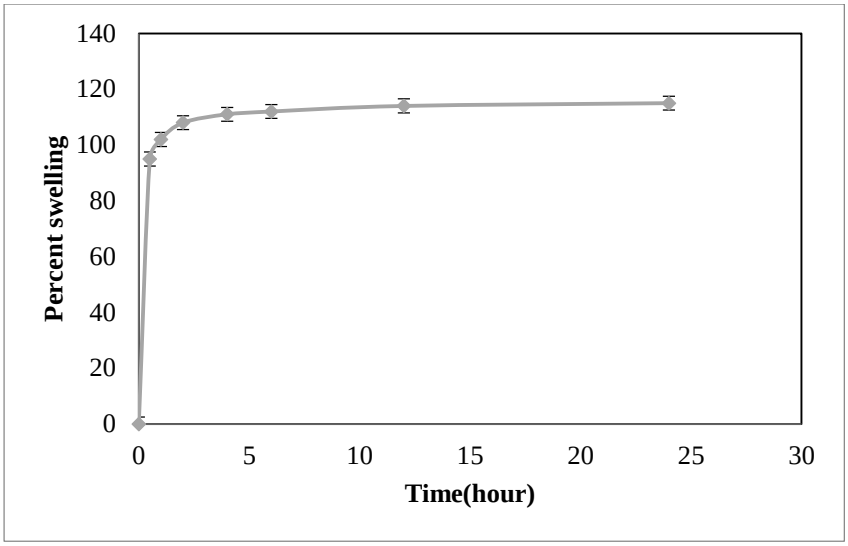

Fig. 3. Percent swelling of GPA-04 impregnated with Ag-NPs film at different time points

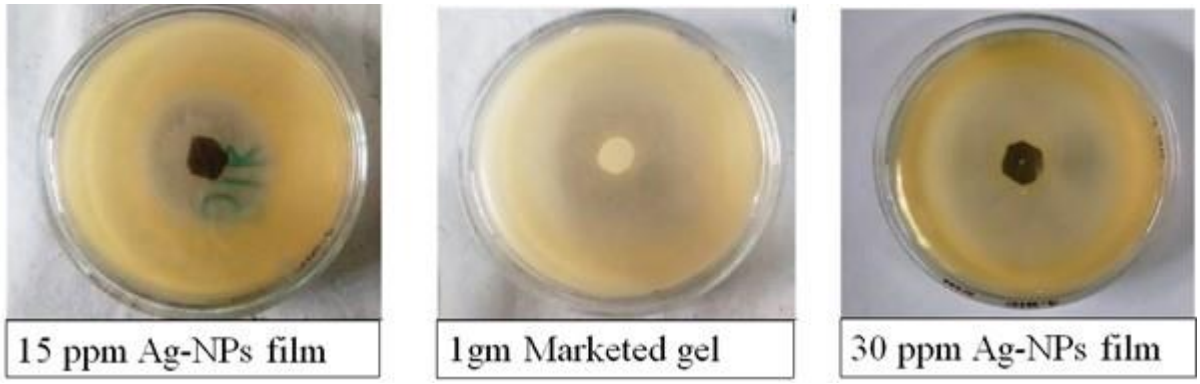

Fig. 4(a). Zone of inhibition for GPA-04 impregnated with Ag-NPs films against *S. aureus*

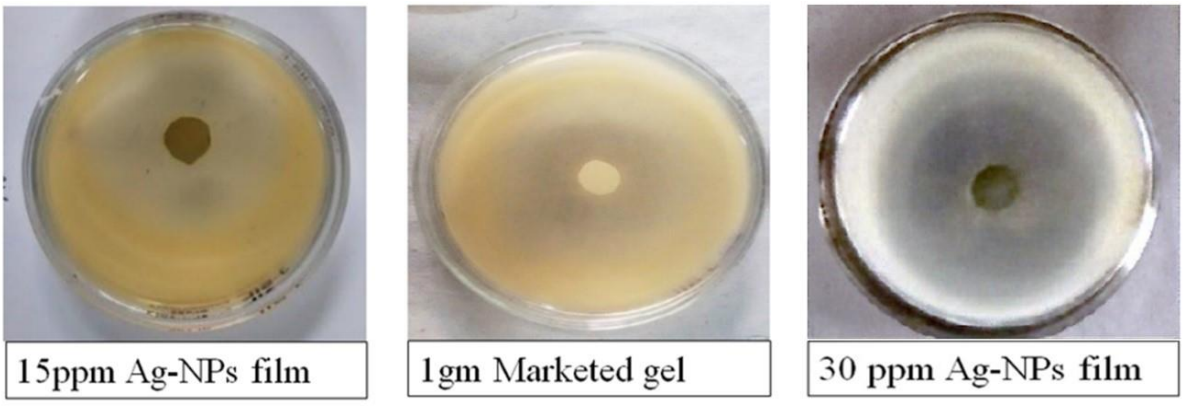

Fig. 4(b). Zone of inhibition for GPA-04 impregnated with Ag-NPs films against *E. coli*