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"Smart" polymer enhances the efficacy of topical antimicrobial agents

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ABSTRACT

The delivery of antimicrobial agents to surface wounds has been shown to be of central importance to the wound healing process. In this work, we prepared film forming wound care formulations containing 3 polymers (FTP) that provide broad-spectrum antimicrobial protection for prolonged periods. FTP formulations comprises of a smart gel matrix comprising of pH-degradable and temperature responsive polyacetals (smart polymer) which allow for the FTP films to be hydrophobic at room temperature, preventing accidental rubbing off, and hydrophilic at lower temperatures, allowing for easy removal. Two FTP smart-antimicrobial films were evaluated in this work: FTP-AgSD (Silver sulfadiazine actives), and FTP-NP (Neosporin actives). The *in vitro* and *ex vivo* antimicrobial efficacy studies show that FTP-AgSD films are significantly more effective for longer durations against *Staphylococcus aureus (3 days)*, *Candida albicans (9 days)* and *Pseudomonas aeruginosa (4 days)* when compared to the cream formulations containing antimicrobials. FTP-NP films showed significantly improved antimicrobial activity for a minimum of 3 days for all pathogens tested. Moreover, when tested *ex vivo* in porcine skin, FTP-AgSD and FTP-NP showed average improvements of 0.89 $\log_{10}$ and 1.66 $\log_{10}$ respectively over standard cream counterparts. Dermal toxicity studies were carried out in a rat skin excision model which showed a similar wound healing pattern to that in rats treated with standard cream formulations as represented by reduction in wound size, and increase in wound healing markers.

1. Introduction

Microbial infections are a major cause of complications in patients with burn wounds, chronic decubitus ulcer wounds and surgical site wounds [1–4]. Burns and decubitus ulcers are considered to be two of the most serious skin injuries [5,6]. Burn and ulcer sites proliferate bacteria and fungi in eschar and underlying viable tissues, making them increasingly susceptible hosts for further bacterial colonization [7].

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Therefore, the aim of wound management and therapy is prevention of infection to support epithelization and accelerate the healing process [8].

Topical therapies have recently become the essential treatment approaches for severe burn and chronic decubitus ulcer wounds to control infection in patients where traditional dressings approaches have failed [9]. Tropical therapies create moist, clean, and warm environment allowing epithelial cells to migrate to the center of the wound, facilitating wound contraction and accelerating the wound healing process [10,11]. Another important advantage of topical treatment over systemic treatment is the delivery of a high concentration of antimicrobials at the site and reduction in systemic cytotoxicity [12]. Silver salts, especially silver sulfadiazine (AgSD), have been regarded as the gold standard in treatment of burn wounds for over 40 years [2,13]. AgSD combines the inhibitory action of silver salts along with the antimicrobial and wound healing effects of sulfadiazine [14]. More recently, AgSD has been shown effective in the epithelization of burn tissue [15] and continues to be the standard of care against which all other wound care treatments are measured.

In topical wound care, AgSD is primarily used in a hydrophilic cream base (Cream-AgSD) or dressing and applied to burn and other surface wounds [16]. Recently, transparent film dressings [17] composed of polyurethane or co-polyester, and hydrogels such as alginates [18], chitosan [19] and hyaluronic acid [20] based biomaterials containing AgSD formulations, have been used as carriers. These dressings possess a unique micromorphology with distinguished physical and mechanical properties that aid in the delivery of antibiotic agents to the wound surface [21,22]. Common disadvantages of these biomaterials are, (1) they can be easily removed after application on the wound surface; thus need to be reapplied daily, which causes severe discomfort and pain for burn patients (especially in the case of second and third degree burns), and (2) AgSD incorporated in commercially available hydrophilic base or hydrogel polymers is released at a faster rate to the wound surface and can interfere with the wound healing process [23]. This study discusses the use of a film forming triple polymer-gel matrix (FTP) containing a novel, polyacetal-based polymer ("smart" polymer). These polyacetal polymers are acid degradable and exhibit a lower critical solution temperature (LCST) in aqueous solutions [24] that is highly tunable. Furthermore, it was shown that precise main-chain functionality can be obtained for polyacetals by simple substitution of the monomer utilized in the polymer synthesis [25,26]. These unique features allow for use of polyacetals as a versatile smart delivery vehicle for use in topical (and intravenous) applications. For example, the temperature response may be used to selectively adjust its hydrophilicity in response to ambient temperature, which prevents rubbing off of the polymer matrix and helps moisture retention. Importantly, the degradation of polyacetals allows for controlled release of active ingredients such as AgSD for prolonged periods of time. The FTP polymer-gel matrix also contains chitosan and hydroxypropylmethycellulose-steoroxyether (Sangelose) polymers, each with unique properties. Chitosan is widely known to be a good wound-healing agent [12,27–29] and provides stability to the base formulation. In an aqueous solution, Sangelose exhibits thermal gelation properties and congeals into a viscous, semi-flexible gel, providing essential hydration and spreadability [30].

In this paper, the temperature responsive properties and *in vitro and ex vivo* antimicrobial efficacy of antimicrobial FTP formulations containing polyacetal as a major component were investigated. At temperatures below the LCST, the FTP is hydrophilic, allowing for facile removal by rinsing. At temperatures above this critical temperature, the film acts as a hydrophobic barrier and remains tethered to the dermal surface. Prolonged release of the antimicrobials is achieved by the pH dependent hydrolysis of polyacetals, making these films highly desirable for a variety of drug delivery applications. We demonstrate this unique property of FTP by the incorporation of AgSD and other actives commonly used in wound care (eg: blends of polymyxin B, bacitracin zinc and neomycin sulfate) along with wound healing agents such as Omega-3 fatty acid (Flax-seed oil) which has been reported to have wound healing properties [31,32]. For comparison, we performed head-to-head evaluations of these actives incorporated into FTP vs. commercially available products incorporated into Cream Base or in Band-Aid®.

2. Materials and methods

2.1. Materials

The following chemicals were purchased from Sigma Aldrich and used as received: tetraethylene glycol (TEG, 99%), tri (ethylene glycol) divinyl ether (TEGDVE, 98%), 1,8-Octanediol (OCT, 98%), 1,4-butanediol divinyl ether (BDVE, 98%), tetrahydrofuran (THF, 99.9%, anhydrous), ethyl acetate (99.9%, HPLC grade) and dichloromethane (DCM, 99.9%), triethylamine (Et_3N, 99.9%), sodium sulfate (99.0%), chitosan (M_n = 600 kDa) and pyridinium p-toluenesulfonate (p-TSA, 99%). Propane-1-3-diol (Zemea, DuPont Tate & Lyle), decanediol (Symrise Inc.), white petrolatum (Sonneborn LLC), behentrimonium methosulphate (and) cetearyl alcohol (BTMS, The Herberie), emulsifying wax (Polawax-NF, The Herberie) and Sangelose (Diado Chemical Corp.) were purchased and used as received. Natural emollients, benzyl alcohol (Fisher Scientific, Fair Lawn, NJ), and ethyl hexyl glycerin (Kumar Organics) were used as received.

2.2. Antimicrobials

Silver sulfadiazene (AgSD) was obtained from Napp Technologies Inc. (Hackensack, NJ, USA). Neosporin® analog was prepared by combining Polymyxin B sulfate (PB, ≥6000 USP units/mg), neomycin sulfate (NS, 10 mg/mL in 0.9% NaCl) and bacitracin zinc (BZ, Bacillus licheniformis, ~70,000 U/g) which were purchased from Sigma Aldrich.

2.3. Wound healing/anti-inflammatory agents

Calendula extract (*Calendula arvensis*), aloe gel (*Aloe barbadensis*), rosemary oil (*Rosmarinus officinalis*), and almond oil (*Prunus dulcis*) were obtained from Naturally Australian Products (Santa Cliarita, CA, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Flaxseed oil was obtained from Real World

Nutrition Laboratory Foundation, Pune, India. Micronized Zinc oxide was obtained from Tri-K (Denville, NJ, USA)

2.4. Microorganisms and growth conditions

Staphylococcus aureus (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 27853), *Methicillin-resistant Staphylococcus aureus* (ATCC 33592) and *Candida albicans* (ATCC 11651) were obtained from the American Type Culture Collection (ATCC). Bacterial and *C. albicans* cultures were prepared from stock agar slants in trypticase soy broth (TSB) (BD, Sparks, MD) and Sabouraud dextrose broth (SDB, BD, Sparks, MD) respectively. After 24 h of incubation at 37 °C, all cultures were centrifuged, washed twice with phosphate-buffered saline (PBS) and re-suspended in PBS to a final concentration of 10^8 CFU ml^{-1} for each microorganism.

2.5. Dilution fluid with neutralizers (DE)

DE was prepared by addition of 34 g of KH_2PO_4, 3 g of lecithin (Fisher Scientific, Fair Lawn, NJ) and 10 ml of Tween 80 (Sigma-Aldrich, St. Louis, MO) followed by adjusting volume to 1 L with deionized water (pH adjusted to 7.2 prior to autoclaving at 121 °C). The effectiveness of using neutralizers in the dilution fluid was validated in our previous studies [33] following the method described by ASTM International (ASTM E1054-08, 2013).

2.6. Synthesis of polyacetal and polyacetal-conjugates (smart polymers)

Polyacetals (PA) were prepared as described previously by Koberstein et al. Two types of temperature responsive PA have been described, (1) PA comprising entirely of ethylene oxide and methylene units, and (2) PA containing other functionalities, in what is known as main-chain PA-drug conjugates [26]. We evaluated both of these polymers in our FTP-formulations, (1) Polyacetal (PA): was prepared using previously described methods [24], and (2) Polyacetal-OCT conjugates: were prepared using a modified version of a previously described method [26]. Briefly, PA-OCT was prepared by replacing a percentage of tetraethylene glycol with 1,8-OCtanediol. For example, PA-OCT-80 was prepared by replacing 80% of tetraethylene glycol with 1,8-OCtanediol. Tri(ethylene glycol) divinyl ether was used for the vinyl ether portion of the reaction. The polymers were purified using previously described methods by Koberstein et al.

2.7. Optimization of concentration of PA/PA-OCT in FTP polymer gel matrix

It is initially hypothesized that the percentage of polyacetal composition in the matrix is proportional to the release rate of antimicrobial. In order to test this hypothesis, FTP formulations with varied PA and PA-OCT concentrations (0-20% w/w) were tested against *C. albicans* using the Bandaid transfer method. In this method, commercially available Bandaid was cut into 1 cm × 1 cm and 0.15 ml of FTP formation containing varied amounts of PA and PA-OCT was applied, after which the pieces were placed on agar plates plated with 0.3 ml of 10 [6] CFU ml^{-1} of *C. albicans* and incubated for 18 h. The zone of inhibition was measured. The optimal concentration of polyacetal was chosen for further study.

2.8. Preparation of FTP-polymer matrix for wound care formulations

The film forming triple polymer (FTP) was prepared by mixing 10.0% PA-OCT, 0.9% chitosan, and 0.2% Sangelose® in emollient solvents (1% decanediol and 1% ethylhexyl glycerin, 4.0% 1,3 propanediol). In addition, 4.0% petrolatum as thickener, 2.5% sorbitan monolaurate as emulsifier, 0.1% rosemary oil as antioxidant and 0.05% Chlorhexidine gluconate as preservative was added and made to 100% with water. The pH was adjusted to 7.5-8.0. Another control formulation without polyacetal (FDP) was prepared to determine the effect of polyacetal on the release of the active ingredients. The FDP polymer matrix contained all the ingredients except polyacetals.

FTP-AgSD and FTP-NP antimicrobial formulations were prepared as follows,

- FTP-AgSD (in FTP gel matrix): 1.0% AgSD, and wound healing agents (calendula oil, zinc oxide and aloe gel, flax seed oil as Omega-3 source) were added and mixed.
- FTP-NP: polymyxin B sulfate 0.064%, neomycin sulfate 0.35% and bacitracin zinc 0.70% as actives (NP) in FTP gel matrix containing wound healing agents as in FTP-AgSD.
- Cream-AgSD was prepared by addition silversulfadiazene and wound healing agents in a hydrophilic cream base.
- Neosporin Original Ointment containing polymyxin B sulfate 0.064%, neomycin sulfate 0.35% and bacitracin zinc 0.70% as actives was purchased from the local pharmacy.
- Commercial-AgSD (Dr. Reddy Labs) was obtained from the local pharmacy.

2.9. Physical properties of FTP-antimicrobial gel matrix

2.9.1. Effect of temperature in the retention of FTP-AgSD during rinsing with water

In order to demonstrate the temperature response of the film formed by FTP at room temperature, 0.2 ml of FTP-AgSD was spread on a 5 cm × 5 cm glass slide and rinsed with water (at 10 °C & 25 °C) at a flow rate of 10 ml s^{-1}. The surface of the glass slide was blotted after each wash and imaged at regular intervals. Cream-AgSD was similarly rinsed for comparison. The AgSD film remaining on the slide was observed (Fig. 2).

2.9.2. Retention of FTP-AgSD and antimicrobial activity after rinsing with water at room temperature: (**ex vivo** *pigskin method*)

FTP formulations were applied on a 5 cm × 5 cm pig skin at 25 °C and the above experiment was repeated. After washing, the pigskin was allowed to dry and subsequently placed on an agar plate seeded with 10^8 CFU ml^{-1} S. aureus and incubated overnight. Cream-AgSD was similarly rinsed for comparison. The colonies under the pigskin were counted and $\log_{10}$ reduction from control was calculated.

2.9.3. Retention of FTP-NP and antibacterial activity in agar plate after rinsing with water at room temperature (plate method)

In order to determine the retention of efficacy of FTP-NP formulations, fresh agar plates were plated with 0.3 ml of FTP

formulations and commercial NP ointment (PBS was used as control) and allowed to dry for 1 h. The plates were then inoculated with 0.3 ml of 10^8 CFU ml^{-1} of bacterial cultures *S. aureus*,*P. aeruginosa* and *C. albicans,* (10^6 CFU ml^{-1} for *C. albicans*) which were used as the test organisms, and incubated for 18 h (Plate A). After incubation, 5 ml of PBS was spread on the plate for 1 min and collected into a culture tube. To the same tube, 5 ml of DE neutralizing broth was added immediately and mixed to neutralize any actives. The samples were serially diluted as necessary and plated (Plate B). The colonies were counted and $\log_{10}$ reduction was calculated with respect to control. Plate A was rinsed with sterile saline and re-inoculated with the same inoculum and the process was repeated until the $\log_{10}$ growth of the FTP samples were comparable to that of control.

2.10. *Biological properties of FTP-antimicrobial gel matrix*

2.10.1. **In vitro** *antimicrobial efficacy of FTP-AgSD and FTP-NP evaluated by zone of inhibition (well plate method)*

Agar plates were seeded with 0.3 ml of 10^8 CFU ml^{-1} bacterial cultures (0.3 ml of 10^6 CFU ml^{-1} for *C. albicans*) and allowed to dry for 1 h in the incubator. Using a sterile cork-borer, 0.7 cm diameter circular agar pieces were removed. The resulting well was filled with 0.3 ml the test samples and incubated overnight. The zone of inhibition was measured after incubation for 18 h. Zone of inhibition of Commercial-AgSD Cream and Neosporin (NP) Cream were also tested for comparison.

2.10.2. **In vitro** *antimicrobial efficacy evaluated by zone of inhibition (pigskin method)*

Sterile pigskin was cut in to 1 cm × 1 cm square pieces and 0.1 ml of antimicrobial formulations were applied on their surface. The pigskin was incubated for 30 min. for the film to completely dry and placed on agar plates plated with 0.3 ml of 10^8 CFU ml^{-1} bacterial cultures (0.3 ml of 10^6 CFU ml^{-1} for *C. albicans*). The plates were incubated overnight and the zone of inhibition was measured after incubation for 18 h. Zone of inhibition of Commercial-AgSD Cream and Neosporin (NP) Cream was also tested for comparison.

2.10.3. **Ex vivo** *antimicrobial efficacy of FTP-antimicrobial topical gels in pig skin infected with bacteria*

The *ex-vivo* porcine skin method was carried out according to ASTM E2897-12. Pre-prepared circular porcine skin (4.1 cm diameter) was adhered to a plastic base and sterilized using 35% ethyl alcohol. The skin was washed with running deionized water for 30 s and then soaked in phosphate buffered saline (PBS) for 20 min to remove alcohol completely. For inoculation, 50 μL each of 10^7 CFU ml^{-1} bacteria was applied on the surface of the pigskin and spread evenly. The skins were then incubated for 20 min for bacterial absorption. For test sample, 0.2 ml of FTP formulations were spread evenly with glass spreader until a uniform layer was obtained. PBS was used as the control and was applied on the porcine skin in the same manner. The samples were allowed to incubate for 2 h and 4 h.

A circular cylinder (2.2 cm internal diameter) was placed over pigskin and 1 ml of DE added into the cylinder. The skin sample was scrubbed with sterile scraper for 15 s and 9 ml of DE was added into the cylinder. The DE was mixed inside the cylinder and transferred to sampling tube together with remaining bacteria on the surface. The samples were serially diluted as required, spread over agar plates with a spreader and incubated for 18 h. The colonies were counted and the $\log_{10}$ reduction values were determined with respect to the control growth (PBS). The samples were tested in triplicate for each experiment and all experiments were performed three times.

All *in vitro* and *ex vivo* experiments are conducted in triplicates and at least four such triplicates (total 12 groups) were used to determine statistically relevant results.

2.11. *Dermal toxicity in rat excision wound model*

Dermal toxicity was determined by observing the effect of FTP-AgSD on wound healing in excised rat skin wounds. Wound healing was evaluated by the reduction in wound size, as well as an increase in wound healing markers, the latter assessed by histopathology of wound tissues.

2.11.1. *Animals*

Healthy adult male Wistar rats (200–230 g) were obtained from the Bharati Vidyapeeth (deemed to be university), Central Animal House, Medical College Pune, India. The animals were housed in solid bottom polypropylene cages and were kept at 24 °C ± 1 °C, with a relative humidity of 45–55% and 12:12 h dark/light cycle. All animals were kept under pathogen-free conditions. The animals had free access to standard pellet chow (Nutrivet Life Sciences, Pune, India) throughout study period. They had access to filtered water. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Bharati Vidyapeeth (Deemed to be University), Medical College, Central Animal House, Pune-411043, Maharashtra State, India (Approval no. BVDUMC/l880/2018/002/009).

2.11.1.1. Excision wound model. Excision wounds were used for the study of rate of contraction of wound and epithelization [34]. Animals were anaesthetized with ketamine (80 mg/kg, i.p), shaved and an impression of size 500 mm [2] and 2 mm depth was made on the back of neck. Excision wounds were made by cutting out layer of skin from the shaven area. Hemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. The entire wound is left open. The progressive changes in wound area were measured on predetermined days i.e., day 8, 12, 16 and 20 post-treatment.

Animals were divided in following four groups:

1 Vehicle control animals: received injury for wound formation but do not receive any topical treatment.
2 Commercial-AgSD Cream
3 Cream-AgSD
4 FTP-AgSD

Wound contraction was calculated as percentage of the reduction in original wound area size (Fig. 7).

2.11.1.2. Histopathology. Skin tissue samples were collected on 4th, 8th, 12th and 20th day from each group. Skin tissues were

fixed in 10% formalin solution. The tissues were trimmed and processed routinely by Alcohol-Xylene method as per standard protocol. The processed tissues were then embedded in paraffin wax and sections were cut at 3–5 μm and slides were prepared. The slides were stained by Hematoxylin-Eosin staining method and mounted with DPX mountant and observed under microscope. Sections were analyzed and scored as mild, moderate and severe for various wound healing parameters/process.

2.12. Statistical analysis

In vitro & ex vivo: Differences between the means of the data sets corresponding to FTP films and their commercial equivalents for one-day well plate studies and ex-vivo studies were analyzed statistically using a Welch's t-test after conducting an F-test of equality of variances. The threshold for significance was set at P, one-tail <0.05.

All animal study results were expressed as Mean ± SD. Data analysis was carried out by GraphPad Prism 5.0 software (GraphPad, San Diego, CA). Statistical comparisons were made between treated groups and wound control animals. Data was analyzed using two-way repeated analysis of variance; Bonferroni's multiple range test was applied for post hoc analysis. A value of $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Synthesis and characterization of polyacetal and polyacetal conjugates (smart polymers)

PA and PA-OCT polymers were characterized using gel permeation chromatography to determine molecular weight and polydispersity index (PDI). PA_{4020} and PA-OCT had M_n of 12.40 kDa and 12.28 kDa with PDI 2.51 and 2.87, respectively. The cloud point temperatures for both polymers were measured using UV/Vis spectroscopy. The cloud point temperatures for PA and PA-OCT were 8 °C and 14 °C, respectively and were consistent with previously published work by Koberstein et al.

3.2. Optimization of PA polymer and its concentration in FTP polymer gel matrix

The pH of chronic wounds is known to be within the range of 7.1–8.9. However, as the wound healing process begins, the pH tends toward neutral and subsquently to an acidic pH range of 5.4–5.6 [35]. FTP formulations are designed for this wound environment as PA_{4020} and PA_{OCT} are known to undergo acid hydrolysis at pH below 7.0 [24] previous work shows that rate of hydrolysis correlates directly with the pH of the environment [24,26]. We hypothesize that the degradation of PA_{4020} and PA-OCT allows for the migration of antimicrobials and degradation products to the surface of the wound. The rate of degradation may determine the concentration of antimicrobials at the wound site at any given time, further correlating to antimicrobial efficacy.

To determine the relative rates of efficacy, we have studied degradation behavior and antimicrobial efficacy using FTP-AgSD formulations of varied concentrations of PA_{4020} and PA-OCT applied to commercial Bandaid (Fig. 1). In the absence of the PA polymer (FDP), antimicrobials rapidly migrate to the surface of the wound largely aided by chitosan (group 0% PA in Fig. 1). However, due to the rapid migration, the overall drug release is terminated within a period of 2–3 days. Similar behavior is observed for PA concentrations less than 10% which appears to be insufficient to influence bulk properties. As the concentration of PA in the formulation is increased, the duration of antimicrobial efficacy increases gradually. Therefore, the amount of PA can be fine-tuned to meet the specification of the end-use.

For PA_{4020} an antimicrobial duration of 6–7 days is observed at a maximum concentration of 20%. To further improve antimicrobial efficacy, a PA-OCT conjugate was prepared. PA-OCT contains a mixture of tetra(ethylene) glycol, tri(ethylene) glycol divinyl ether and antimicrobial diol, 1,8-OCtanediol (PA main-chain conjugate). By incorporating antimicrobial diols into the polymer system, significant improvement in the antimicrobial efficacy is achieved. At a concentration of 10%, PA-OCT shows significantly improved antimicrobial efficacy ($p < 0.05$) when compared to 10% PA_{4020} and was comparable to that of 20% PA_{4020}. It is therefore apparent that fine control over the duration of the antimicrobial release can be attained by fine-tuning of the type of polyacetal and its concentration in the blend. For the wound care formulations, an ideal duration of release is 5–7 days [7,36]. Therefore, for all subsequent formulations and testing, 10% PA-OCT polymer was used as the optimized concentration of the PA polymer.

3.3. Physical properties of FTP antimicrobial gel matrix

The premise of incorporating PA-OCT a release agent in the FTP formulations is to take advantage of the cloud point

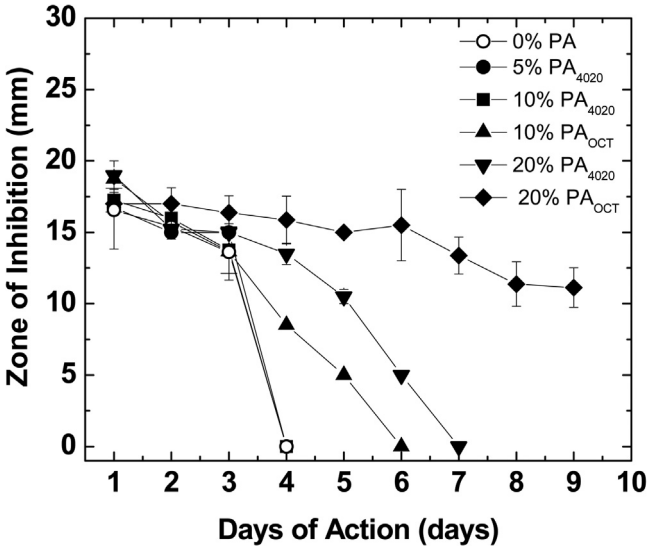

Fig. 1 – Effect of polyacetal polymer concentration (in FTP) on the release of antimicrobial agent (AgSD) characterized by zone of inhibition for optimization of polymer concentration. The organism tested was *C. albicans*. Groups tested were 5% PA_{4020}V (solid circle), 10% PA_{4020} (solid square), 10% PA_{OCT} (solid triangle), 20% PA_{OCT} (solid diamond) and 0% polyacetal (FDP, open circle).

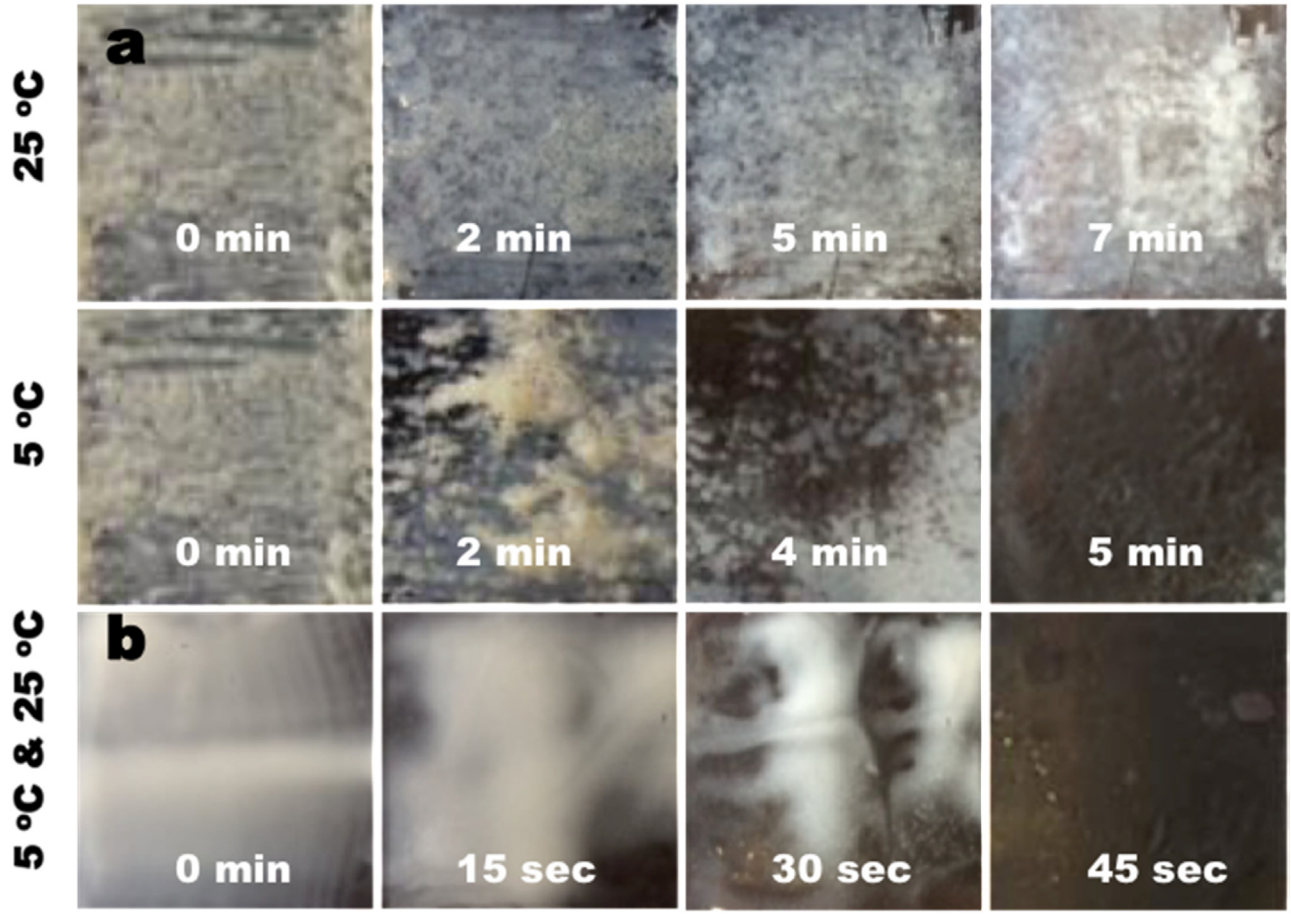

Fig. 2 – Retention of AgSD incorporated in (a) FTP-AgSD and (b) Cream-AgSD when applied on a glass substrate. The study was conducted by rinsing the above two groups with deionized water at 10 ml/s for temperatures above and below the LCST (10 °C). The off-white residues seen were identified as AgSD.

temperature (LCST) reported by Koberstein et al [24], which renders the bulk antimicrobial film hydrophobic above a predesignated temperature (PA_{4020} 8 °C, PA-OCT 14 °C). For wound care applications, above the CPT, the bulk FTP formulation forms a transparent film that is hydrophobic, which prevents the film from being rubbed away. This feature is especially important for bedridden patients who have difficulty in lying on their back, during the period of treatment. Fig. 2 shows a macroscopic view of glass slides treated with FTP-AgSD and rinsed for 0–7 min with running water at a flow rate of 10 ml s^{-1}. The results show that above the CPT (at 25 °C), AgSD is retained on the surface for more than 7 min, despite the harsh conditions of a constant-flow system. However, upon cooling the flow system below the CPT (at 5 °C), a complete removal of the film was observed within 5 min. This result was compared to a rinse experiment conducted with Cream-AgSD, which showed that the cream could not withstand the flow rate for more than 30 s at any temperature.

In order to determine the residual antimicrobial efficacy, the washing procedure was repeated in *ex vivo* pigskin treated with the FTP formulations (Fig. 3). After the wash cycle AgSD remaining on the pigskin was determined by infecting the pigskin with *S. aureus* and determining the bacteria remaining on the skin as compared to pigskin containing no AgSD (control). The results show that after several cycles of continuous washing, 10% PA-OCT and 15% PA-OCT FTP solutions show significantly higher $\log_{10}$ reduction as compared to Cream-AgSD. Cream-AgSD rapidly loses its antimicrobial efficacy during the wash cycle. We deduce that the temperature response of the FTP film aids in the retention of the formulation in the pigskin, prolonging the efficacy of the antimicrobial properties of the film.

The retention of antimicrobial efficacy was further investigated using the plate method to mimic the prophylactic efficacy of the FTP film. FTP-NP was used as the antibacterial formulation in this experiment. In a clinical setting, chronic wounds are first cleaned with ethyl alcohol to prevent infection. Wound care formulations are subsequently applied and covered with gauze. Infections typically occur during the initial cleaning period where the wound is exposed to bacteria from the environment. To mimic this situation, the wound care formulation (FTP-NP) was first applied to the agar plates, and the infection was introduced subsequently. Following incubation, the film was washed with drug neutralizing DE broth and the bacteria on the plate was extracted and quantified. Analogous to a clinical setting,

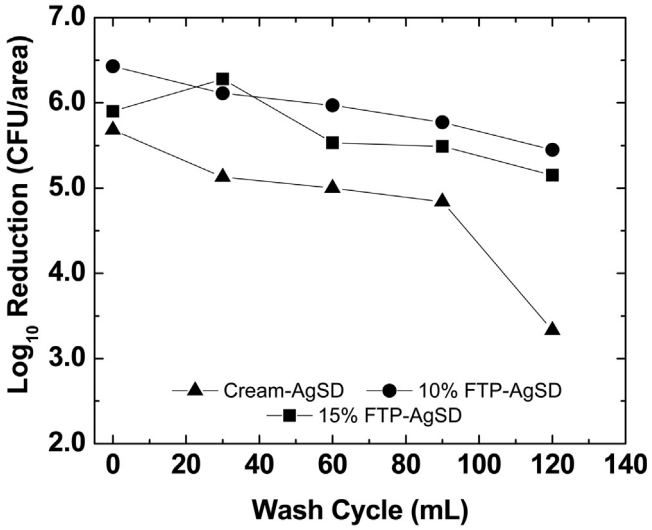

Fig. 3 – Prolonged antimicrobial efficacy of 10% PA-OCT FTP-AgSD (circle) and 15% PA-OCT. FTP-AgSD (square) was compared to Cream-AgSD (triangle) using an *ex vivo* pig-skin model, post washing several cycles with deionized water. Test organism was *S. aureus*.

increased amounts of bacteria were introduced and the process was carried out over several days. The results show that, (Fig. 4) for all pathogens tested the FTP-NP film formulation retained a higher $\log_{10}$ reduction for prolonged periods of time. For *S. aureus*, an average improvement of $\log_{10}$ reduction of 4.56 ± 1.19 was obtained over Cream-NP. Similarly, for *C. albicans* and *P. aeruginosa*, average improvements of 3.24 ± 0.39 and 0.75 ± 0.17 over Cream-NP were obtained. This reinforces the hypothesis that the FTP-NP film-forming gel has the ability to act as a preventative measure against bacterial infection.

3.4. *Broad spectrum antimicrobial activity of FTP compositions*

To establish the full antimicrobial spectrum, FTP formulations (FTP-AgSD and FTP-NP) were challenged against four prevalent pathogens. The abundance of the four pathogens, *S. aureus* (6.5–37.6%), *P. aeruginosa* (17.0–51.5%), *C. albicans* (1.3–22.46%), *MRSA* (0.2%) chronic in wound infections has been well documented [37]. Despite the low abundance, *MRSA* is particularly of concern due to antimicrobial resistance. In the first test (Fig. 5), *initial* antimicrobial efficacy using a well plate method for FTP-AgSD was compared to commercially available Cream-AgSD, the current standard of care for burn wound creams. Similarly, FTP-NP was compared to commercially available Cream-NP. The results show that FTP-AgSD has comparable or better initial efficacy to Cream-AgSD. In the pathogens tested, *S. aureus* and *MRSA* showed a statistically significant increase in the zone of inhibition while *C. albicans* and *P. aeruginosa* showed comparable efficacy ($p < 0.05$). A possible explanation for this result could be the initial burst of AgSD in both FTP-AgSD and Cream-AgSD responsible for the initial bactericidal activity. Similarly, the initial release study of FTP-NP exhibits

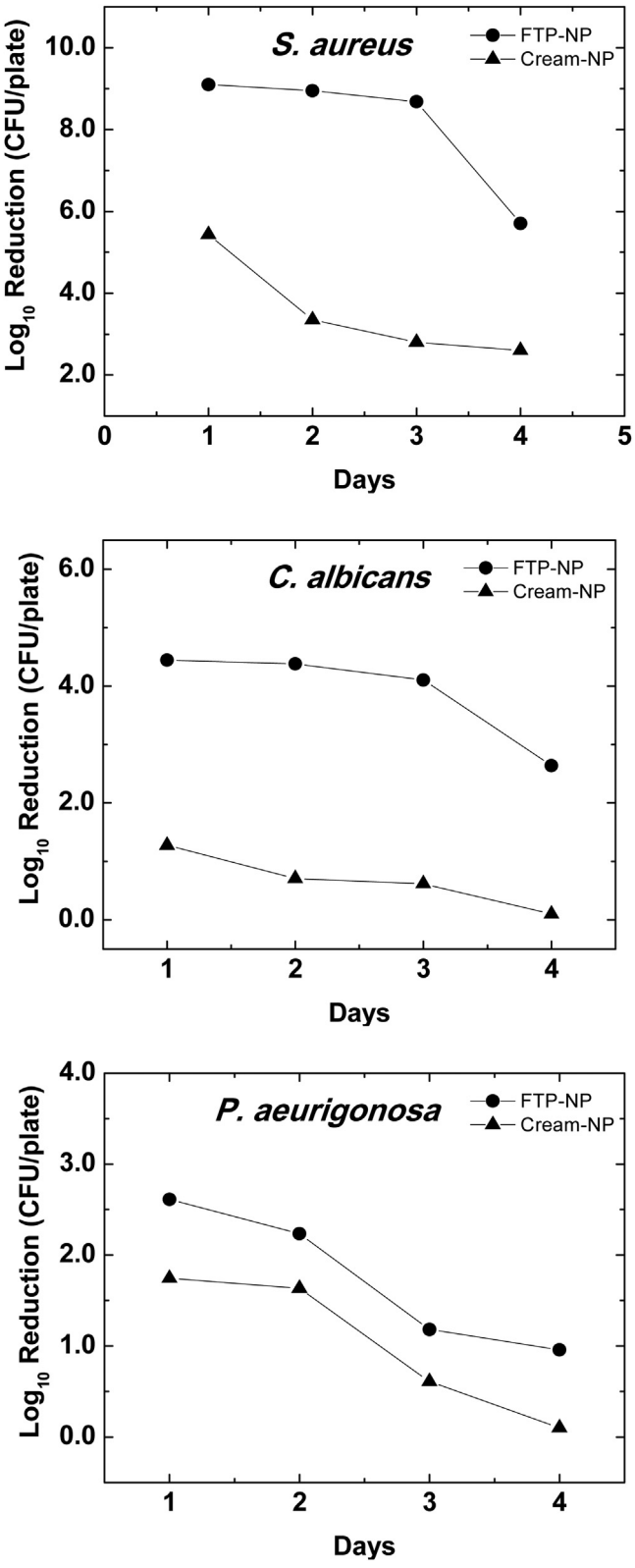

Fig. 4 – Substantive activity of FTP-NP vs. Cream-NP performed using the plate method with daily re-inoculation of bacteria.

significantly higher antimicrobial efficacy in all cases tested, except for *MRSA*, in which FTP-NP shows equal efficacy to Cream-NP ($p < 0.05$). We observed significant Neosporin resistance in *P. aeruginosa* and *C. albicans* in this study.

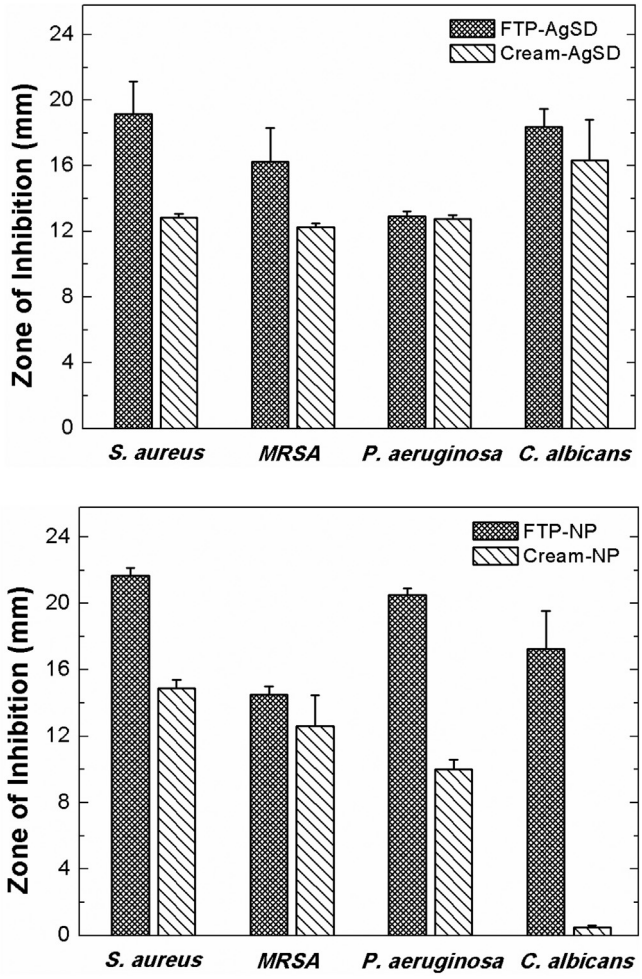

Fig. 5 – Initial release of antimicrobials in (a) FTP-AgSD vs. Cream-AgSD and (b) FTP-NP vs. Cream-NP in a one-day well plate study against four common pathogens.

Particularly in the case of *C. albicans*, largely insignificant antimicrobial activity was observed in Neosporin. However, by incorporating Neosporin actives into the FTP polymer base, its antimicrobial activity was significantly enhanced. We therefore deduce that the carrier plays a vital role in the delivery of antimicrobials to the site of the wound, regardless of the antimicrobial agent.

3.5. *Sustained antimicrobial activity of FTP compositions*

S. aureus, *C. albicans* and *P. aeruginosa* are also among the most prevalent microorganisms found in diabetic foot ulcers [38,39]. Therefore, sustained activity against these bacterial targets during *in vitro* studies would indicate the clinical efficacy of FTP-antimicrobial gels. The duration of antimicrobial activity was determined by daily transfer of the thinly sliced porcine skin onto freshly seeded agar plates and measuring their zones of inhibition (Fig. 6). In this study, FTP-AgSD was compared to commercially available Cream-AgSD. For *S. aureus*, FTP-AgSD retained its antimicrobial efficacy for 3 days, while Cream-AgSD showed efficacy for 2 days. Furthermore, a significant increase in duration of FTP-AgSD antimicrobial efficacy was observed for *C. albicans*, which was active for 9 days. In contrast, Cream-AgSD showed efficacy for 4 days. Similarly, for *P. aeruginosa*, FTP-AgSD improved the duration of efficacy by at least 1 day when compared to Cream-AgSD.

The study was repeated for FTP-NP and compared with Cream-NP, BandAid® and RiteAid® commercial antimicrobial wound dressings. For all studies conducted, commercially available BandAid-Plus-Antibiotic® had extremely low antimicrobial efficacy. RiteAid® adhesive bandaid had efficacy comparable to Cream-NP for *S. aureus*, but had a maximum duration of only two days. FTP-NP and Cream-NP also showed comparable efficacy for *S. aureus*. However, for *C. albicans* and *P. aeruginosa*, FTP-NP showed significantly improved antimicrobial efficacy. It is important to note that Cream-NP had no antimicrobial efficacy against *C. albicans*. The results bolster our initial hypotheses that, (1) the migration of the antimicrobial actives in FTP during the initial contact with the agar surface is significantly higher than its cream form, and (2) antimicrobials embedded in FTP have longer release times than the cream form.

3.6. *Ex vivo antimicrobial efficacy of FTP-antimicrobial gels (ASTM E2897-12)*

As a close substitute for human skin conditions, porcine skin was used to further confirm the antimicrobial efficacy of FTP compositions. The study was designed to demonstrate the bactericidal effect of FTP-gels in short contact times. Wound healing typically occurs during a period of 4–10 days [40–42]. However, it has been shown that the process of healing can be compromised if the wound is infected [41]. Therefore, control of infection is crucial aspect wound healing. At 2 and 4 h of contact time, FTP-AgSD and FTP-NP showed significantly higher $\log_{10}$ reduction from control (PBS), than Cream-AgSD and Cream-NP for all pathogens tested ($p < 0.05$) (Table 1). This result can be attributed to the rapid release of antimicrobials during the first 24 h of contact as well as increased retention of antimicrobials on the skin. At a longer contact time of 4 h, a further increase in the $\log_{10}$ reduction from control was observed due to increased contact time. This $\log_{10}$ reduction was significantly higher than that observed in the Cream forms. The results confirm the hypothesis that the FTP formulation significantly prolongs the efficacy of the antimicrobials.

3.7. *Dermal toxicity of FTP-AgSD formulations using excision wound model in rats*

Wound healing potential of silver sulfadiazine has been studied extensively in various animal models [43]. Adhya et al., reported topical applications of silver sulfadiazine which show reduced adverse effects than that of conventional treatment and does not interfere with wound healing [44]. However complete wound healing takes a considerably longer. Wound healing includes various biochemical and cellular events and are mainly divided in three major phases namely: inflammation, proliferation and remodeling. Wound healing involves immune cells in its resolution, hence essential nutrients such as amino acids, minerals, and fatty acids plays key role in wound healing [45]. Role of omega-3 fatty acid in

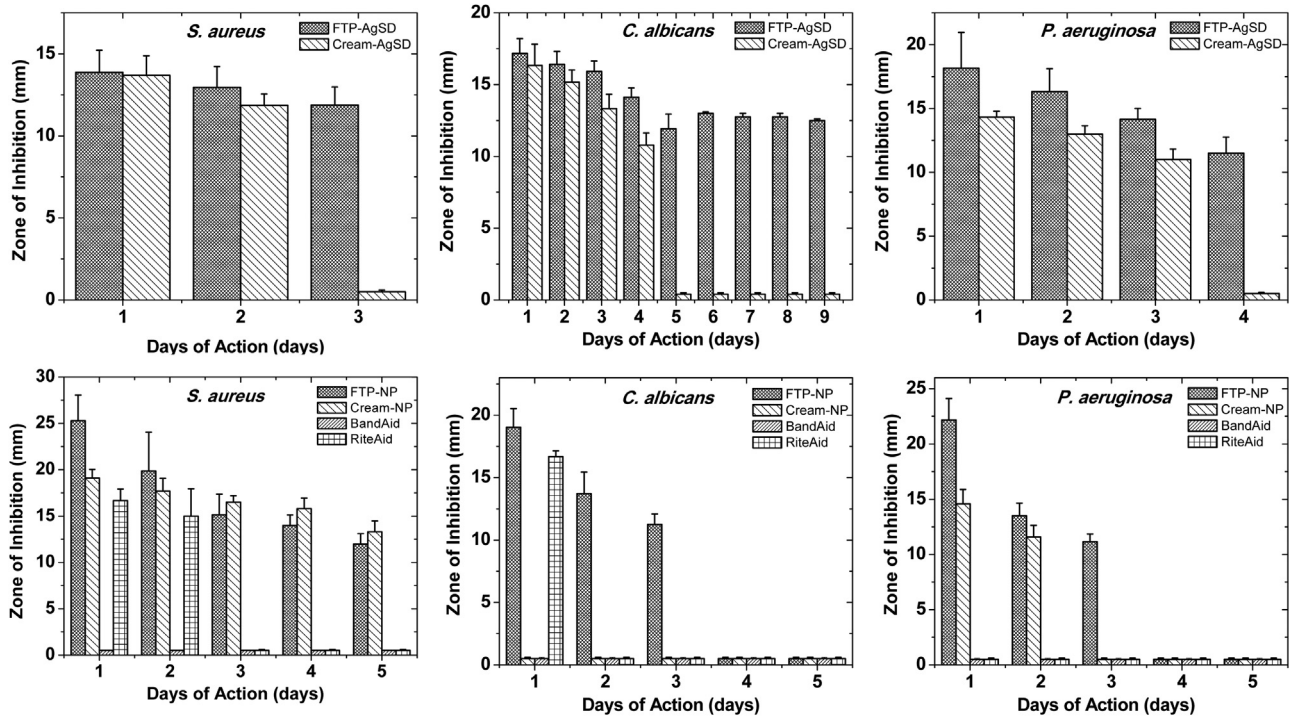

Fig. 6 – Comparison of duration of AgSD release quantified through zone of inhibition in (a) *S. aureus*, (b) *C. albicans* and (c) *P. aeruginosa*. The AgSD release of FTP-AgSD (circle) is compared to Gel-AgSD (square) and Cream-AgSD (triangle).

inflammation is well studied [31,32]. Lewinska et al., reported effect of flaxseed/linseed oil on wound healing in in vitro studies [46]. Alpha-Linolenic acid (ALA) rich treatments exhibited potent wound healing activity. Therefore, the effect of Omega-3 in FTP-AgSD as well as Cream-AgSD was additionally explored.

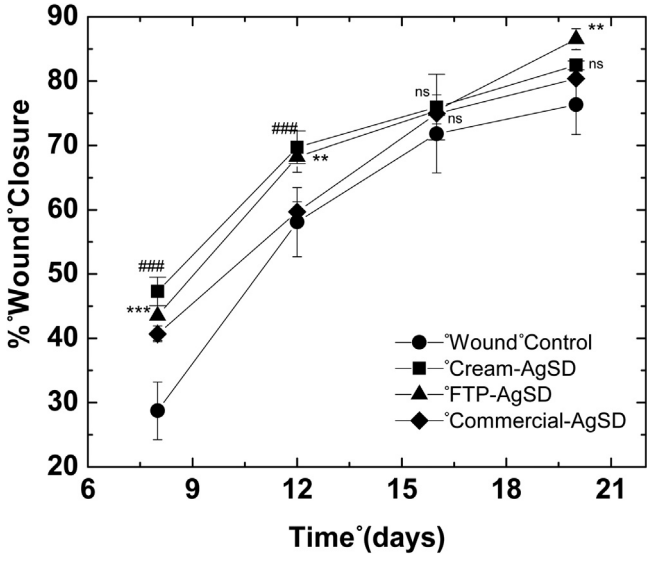

Fig. 7 – Wound contraction comparison on Day 8, 12, 16 and 20 post treatment FTP-AgSD, Cream-AgSD, Commercial-AgSD and wound control (received wound but no treatment). Percentage calculated from original incision.

Wound healing properties of FTP AgSD, Cream-AgSD and Commercial-AgSD were compared on excised rat wounds. The wound area was measured on 0, 8th, 12th, 16th and 20th day in all animals and percentage wound closure was calculated and presented in Fig. 7. The results show that FTP-AgSD gel shows similar or better wound healing properties to Cream-AgSD and Commercial-AgSD groups. Furthermore, FTP-AgSD aids in the wound healing process to heal and contract the wound faster, when compared to control group and Commercial-AgSD groups on day 8 ($p < 0.001$), day 16 ($p < 0.01$) and day 20 ($p < 0.05$). Further, FTP-AgSD showed significant improvement on day 20 ($p < 0.05$) as compared to control group. Similar wound contraction was noted in Cream-AgSD and Commercial-AgSD indicating that FTP formulations are non-toxic, and comparable to Cream-AgSD and Commercial-AgSD.

3.8. *Histopathology*

As summarized in Table 2 and Fig. 8, all three groups, FTP-AgSD, Cream-AgSD and Commercial-AgSD showed a gradual reduction of inflammatory cells during the course of 20 days after excision (post treatment). Vascularization and re-epithelization was observed in the order FTP-AgSD > Cream-AgSD > Commercial AgSD. Control group showed minimal scab formation and mild to moderate re-epithelization were observed. Importantly, based on microscopic examination it appears that rats treated with FTP –AgSD exhibits similar wound healing pattern as that of Cream-AgSD which indicates that this formulation may be safe for topical use. However additional toxicity studies of FTP AgSD need to be conducted to confirm safety of this formulation for human use.

Table 1 – *Ex vivo* rapid-kill results for FTP-AgSD and FTP-NP compared to their cream-base counterparts.

Contact time	Organism	Log_{10} reduction (CFU/ml)		
		S. aureus	*P. aeruginosa*	*C. albicans*
2 h	FTP-AgSD	3.16±0.47	2.26±0.60	2.00±0.30
	Cream-AgSD	1.71±0.18	1.58±0.67	1.48±0.27
4 h	FTP-AgSD	2.83±0.50	3.72±0.13	2.58±0.19
	Cream-AgSD	2.16±1.03	2.86±0.09	1.37±0.01
2 h	FTP-NP	5.53±0.53	3.00±0.05	1.64±0.20
	Cream-NP	3.35±0.27	1.43±0.06	0.42±0.54

Table 2 – Histopathological examination in excision wound model.

	Day	Control	Commercial AgSD	Cream AgSD	FTP AgSD
Inflammatory cells	4	4+	4+	4+	4+
	8	2+	2+	2+	2+
	12	1+	1+	1+	1+
	20	1+	1+	1+	1+
Vascularization	4	0	0	1+	1+
	8	0	1+	2+	2+
	12	0	1+	3+	4+
	20	1+	2+	3+	4+
Fibroblast/reepithelization	4	0	0	1+	1+
	8	0	0	2+	2+
	12	0	1+	3 +	4+
	20	1+	2+	3+	4+

0 None.
1+ Mild.
2+ Mild-moderate.
3+ Moderate.
4+ High.

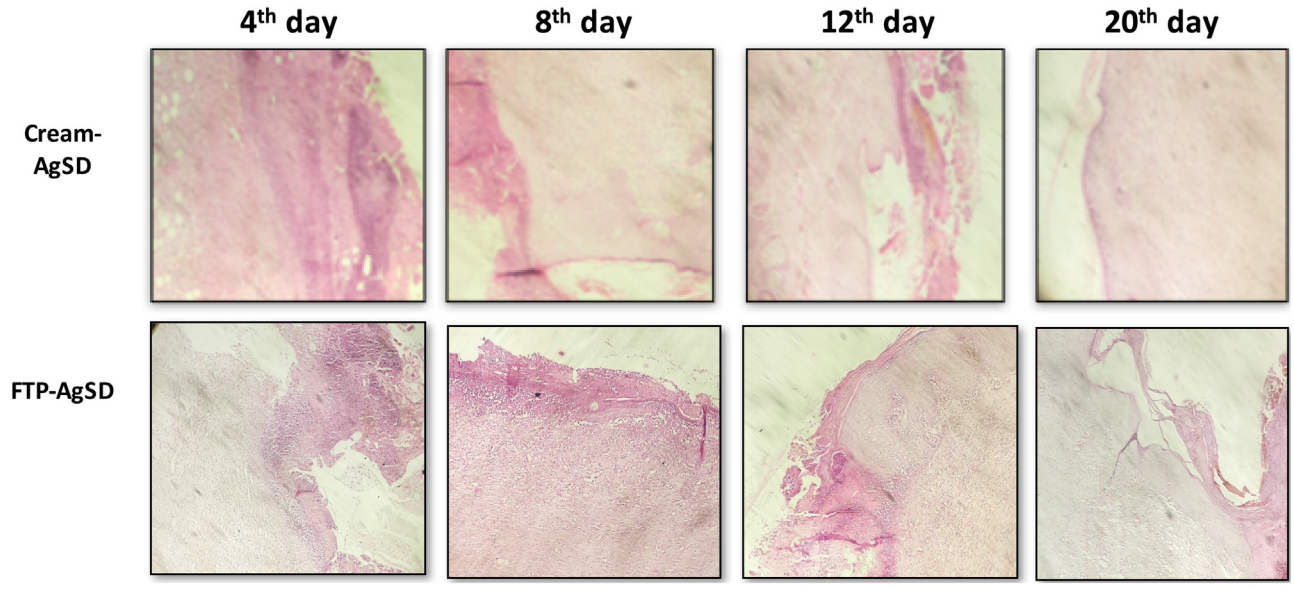

Fig. 8 – Histopathological images for the excision rat model. The model shows increased vascularization and re-epithelization which are markers for wound healing.

4. Conclusion

This work explores the *in vitro*, *ex vivo and in vivo* efficacy of FTP formulations with polyacetal as the matrix base. We discuss two FTP based antimicrobial formulations: FTP-AgSD and FTP-NP, which are designed for use in burns, ulcers and minor wounds. Burn wounds especially require long acting treatments methodologies which are necessary to prevent infection and improve patient comfort. Current treatments include wound dressings, typically containing silver sulfadiazine (AgSD). Wound dressings that require frequent removal and reapplication, which cause significant discomfort to patients. FTP formulations described in this work shows promise in improving the duration of antimicrobial activity during the wound healing process and eliminates the need for wound dressings.

FTP formulations consist of polyacetal as primary component which have recently been identified as a temperature responsive polymer with pH degradability. Remarkably, polyacetal based FTP compositions form a hydrophobic film when applied on the wound surface at room temperature but at temperatures below the room temperature, undergoes a phase transition to become hydrophilic; allowing for facile removal upon cold water rinsing. Thus, unlike standard of care creams, the FTP formulations cannot be otherwise removed or washed off, and daily application or dressing changes, often causing a great deal of pain, may not be necessary.

We have prepared two FTP formulations containing silver sulfadiazine (AgSD) and a combination of neomycin sulfate, bacitracin zinc and Polymyxin B (collectively Neosporin). Broad spectrum antimicrobial studies show that FTP formulations containing AgSD and Neosporin are highly active against common wound pathogens such as *S. aureus*, *MRSA*, *C. albicans* and *P. aeruginosa*. Additionally, FTP formulations have markedly improved duration of efficacy when compared to commercially available creams with same actives. The antimicrobial activity and its duration of efficacy may depend on several factors. However, a main consideration is the antimicrobial migration to the surface of the cream or gel which is then transferred to the surface of the wound. In FTP formulations, acid hydrolysis of the polyacetal polymer provides a medium for drug migration even after initial drug release. For instance, the results show that beyond a threshold of 10% polyacetal there is a significant increase absolute amount of drug released to the surface. This may be due to the loosening of the tightly packed gel to allow interstitial drug migration. We also observe that PA-OCT released significantly more drug than PA_{4020}. A possible explanation could be the ability of humectants such as caprylyl glycols (octanediol) to enhance antimicrobial efficacy of formulations [47,48]. Further study is warranted to fully elucidate the activity enhancing mechanism of FTP formulations.

We have further explored the antimicrobial spectrum of FTP formulations in porcine skin (as a direct substitute for human skin) and in animal models. These two models have further demonstrated that the FTP base significantly improved antimicrobial efficacy when compared to commercial standards. The animal models show a reduction of inflammatory cells, improved in vascularization and re-epithelization and no irritation or toxicity arising from FTP formulations in excised rats that are treated daily for a prolonged period. We continue to explore the dermal toxicity of FTP formulations as a prerequisite for broader applicability of FTP formulations.

Conflict of interest

Authors declare no competing or conflicting interests.

Acknowledgments

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