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RESEARCH PAPER

Drug-Loaded Polymeric Composite Skin Graft for Infection-Free Wound Healing: Fabrication, Characterization, Cell Proliferation, Migration, and Antimicrobial Activity

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ABSTRACT

Purpose A new, injectable, drug-loaded composite graft was developed to enable infection free wound healing.

Methods The graft was fabricated using gentamicin and biomimetic microparticulate scaffolds in gelatin gel and characterized for biologically relevant properties like fluid uptake, evaporative water loss (EWL), water vapor transmission rate (WVTR), Young's modulus and degradation. It was evaluated for drug release, cytocompatibility and antimicrobial efficacy against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Results Graft exhibited fluid uptake of 13.79%, EWL of 60–70% in 10 h, WVTR of 5480.31 g/m^2/d, and Young's modulus as 2.1–10.8 kPa. It exhibited 99.36% degree of crosslinking and a dual degradation behavior wherein, the carrier gel, gelatin, degraded rapidly leaving the microparticulate scaffolds intact. Drug release studies showed a sustained release of gentamicin for 13 days sufficient to inhibit the infection at the wound site. Cytocompatibility assessment of the graft revealed that graft supported cell adhesion, proliferation and migration. The antibacterial efficacy of the graft was assessed using Kirby-Bauer method and time kill assay, wherein results indicated a quick, effective (≥5-log reduction in CFU/ml) and long lasting antimicrobial effect.

Conclusions These results as a whole indicate that the graft represents an effective alternative for infection-free healing of full thickness wounds.

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ABBREVIATIONS

cPMS	biomimetic microparticulate scaffolds
EWL	evaporative water loss
Gel	gelatin crosslinked with glutaraldehyde
Gel-Gen	gentamicin loaded gelatin gel crosslinked with glutaraldehyde
Gel-Gen-cPMS	composite skin graft containing gentamicin and biomimetic microparticulate scaffolds
GTA	glutaraldehyde
HDFs	human dermal fibroblasts
TNBS	2,4,6-trinitro-benzenesulphonic acid
w/o/w	water-in-oil-in-water
WVTR	water vapor transmission rate

INTRODUCTION

Wound healing is a well-coordinated and dynamic process involving the interactions of cells, extracellular matrix and cellular microenvironment to replace the injured tissue with a provisional matrix (granulation tissue) and finally remodel it to form the healthy tissue. To facilitate this process, wound grafts/dressings are used clinically to cover and protect the injured surfaces. An ideal synthetic biological graft should be biocompatible and biomimetic in nature to promote fibroblast proliferation, collagen synthesis, remodeling of injured tissue and thus help in regeneration of healthy tissue (1). It should also maintain a moist healing environment and prevent excessive build-up/loss of

exudates during the healing process (2). Additionally, it should also be bioresorbable to prevent frequent changes which would otherwise harm the underlying vulnerable tissue and cause patient discomfort (3). Bioresorbable film dressings based on lactide–caprolactone copolymers such as Topkin® (Biomet, Europe) and Oprafol® (Lohmann & Rauscher, Germany) are currently available where biodegradation of the film occurs *via* hydrolysis of the copolymer into lactic acid and 6-hydroxycaproic acid however, these dressings lack fluid absorbance and are impermeable to water vapor or gases and cause fluid accumulation on larger wounds (4). Another major attribute of an ideal skin graft should be impermeability to exogenous microorganisms and inhibition of the wound surface flora and bacteria. Hydrogel based grafts fulfill most of these requirements of an ideal skin graft and have been explored in literature for wound healing purpose (5,6).

Repair of the injured tissue proceeds through haemostasis, inflammation, proliferation and remodeling phases. These phases are altered from their normal sequence in the case of infections. If a wound becomes infected, the inflammatory phase becomes chronic; suppressing the regenerative phase. Moreover, the enzymes and toxins generated by the colonizing microorganisms; effect the structural integrity of wounded tissue undergoing healing process alongwith the surrounding skin as well as the skin graft (7). Thus, it is critical to prevent infections in the healing wound bed which could be done by administration of antibiotics. Currently, this is done by systemic administration of antibiotics along with applying the skin graft. However, systemic antibiotic therapy is often associated with development of antibiotic resistant strains and systemic side effects due to larger amount of drugs required to achieve therapeutic concentrations at the infection site. This can be overcome by localized delivery of the antibiotic at infection site using a drug loaded skin graft which can provide the minimum inhibitory concentration of antibiotic in a sustained manner and would be a safer and more effective option (4,8).

Nevertheless, the main challenge in designing a drug loaded skin graft for low molecular weight hydrophilic antibiotics is to control and sustain the drug release at required concentration for desired time period. The local antibiotic should exhibit a considerable initial rate of release to combat the elevated risk of bacterial contamination introduced in the wound during the initial trauma, followed by a sustained release at an effective level to inhibit latent and long term infection (4,9). Other antibiotic eluting devices such as periodontal devices (10) and vascular grafts (11,12) also reported a fast release of the antibiotics. Common strategies that have been described in an attempt to overcome the problem of rapid drug release include the entrapment of the hydrophilic drug within a hydrophobic substance as a means to delay water penetration and outward drug diffusion (13), or enhancement of drug bonding to the carrying matrix (11,14).

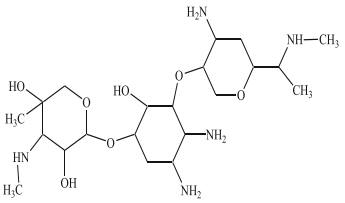
In the present study, gentamicin loaded composite skin graft was developed with the aim that it should provide better and faster skin regeneration and protect the wound from microbial infections. To support the growth of cells; biomimetic, porous, microparticulate based scaffolds are used which were developed by our group and reported elsewhere (15,16). For effective wound healing, migration of dermal fibroblasts is considered to be the initial event and with time, cells should migrate towards the wound margins for its closure and thus heal the wound. TGF-β plays an essential role in cell migration alongwith other cellular functions including epithelization, collagen synthesis with maturation, and matrix deposition (17). TGF-β mimicking nature of cytomodulin and hence its role in wound healing is well reported (18). To obtain these advantages of cytomodulin in the graft, it was coupled on the surface of prepared porous microparticulate scaffolds. Further, to ensure an infection free environment during the healing process, gentamicin was selected as antibiotic agent since it is a broad spectrum antibiotic and effective against a wide variety of gram-negative and gram-positive microorganisms (Table I). To facilitate the delivery of microparticulate scaffolds and drug at the wound site, gelatin was selected as a carrier gel which is biocompatible, completely bioresorbable and non-immunogenic in nature (19). To maintain the required injectability of the graft, crosslinker glutaraldehyde was used. The graft was studied for different gentamicin loadings and amount of scaffolds, characterized and optimized for properties which are of significance in wound healing and evaluated for *in vitro* drug release, cytocompatibility and antimicrobial properties.

MATERIALS AND METHODS

PLGA used for scaffold preparation was synthesized *in house* and characterized by GPC and NMR (MW_n: 75 kDa, MW_w: 94 kDa, PDI: 1.25 and LA/GA from ^{1}HNMR: 2.7/1). Gelatin (Source: bovine skin, Type B < ~Bloom 225 g >) was purchased from Sigma, USA. Gentamicin sulphate was a kind gift from Prof. Avi Domb, Hebrew University of Jerusalem, Israel. All other chemicals and reagents used were of analytical grade.

Human dermal fibroblasts (HDFs; passage 4–6) were harvested from the samples of the healthy human controls and were procured from PGIMER (Post Graduate Institute of Medical Education and Research) Chandigarh, India as per institutional guidelines. Microorganisms *Pseudomonas aeruginosa* (MTCC 424) and *Staphylococcus aureus* (MTCC 3160) were obtained from Institute of Microbial Technology (IMTech), Chandigarh, India. The organisms were grown

Table I Gentamicin: Structure, Physicochemical Properties and Antibacterial Spectrum

Gentamicin ($C_{21}H_{43}N_5O_7$)	Molecular weight (g/mol)	Aqueous solubility	Antibacterial spectrum of activity
	477.6	>1 gm/ml	Effective against a broad range of Gram-negative bacteria including *Pseudomonas, Proteus, Serratia* and Gram-positive bacteria *Staphylococcus*

in nutrient broth culture media (Himedia, Mumbai, India) and were allowed to attain log phase of growth.

Fabrication of Composite Skin Graft

The composite skin graft comprised of two major components i.e. Component 1- cytomodulin coupled porous microparticles (cPMS) to function as scaffolds for cell adhesion and growth and, Component 2- gelatin based gel (Gel) to deliver the antibacterial drug gentamicin (Gen). These two components were formulated in a composite graft crosslinked with glutaraldehyde (GTA).

Component 1

PLGA porous microspheres were prepared by w/o/w method using ammonium bicarbonate as gas foaming agent (15,16). These microspheres were hydrolyzed in the presence of NaOH (0.05 M) followed by cytomodulin (200 ng/ml) coupling onto the hydrolyzed PLGA scaffolds using EDC-NHS as coupling agents. The cytomodulin coupled particles so obtained were freeze dried to obtain cPMS, characterized for physical properties, and evaluated *in vitro* in cell culture studies and *in vivo* in full thickness wound model (20).

Component 2

Gelatin aqueous solution was prepared by dissolving gelatin in hot water (50°C) and cooled to bring this solution at 25°C. Gentamicin was dissolved in minimum volume of purified water and added to gelatin aqueous solution to obtain the desired drug concentrations (2% and 5%w/v) and the final concentration of gelatin in the solution was kept as 15%w/v.

Composite Graft

Weighed amount of cPMS were initially wetted with water to displace the entrapped air followed by addition of gentamicin containing gelatin solution and mixed to form a homogenous system such that desired concentration of gentamicin could be achieved. Cross-linker GTA (0.5%w/v) was added to enable gel formation and to obtain the final composite skin graft. Amount of cPMS and gentamicin in the composite graft were varied (0.1, 1 and 5% w/w) and (2% and 5%w/v) respectively (Table II) and characterized for different biologically relevant properties.

Homogeneity and morphology of the graft was studied by using SEM. For this purpose, the freeze-dried sample of the graft was cut into thin horizontal sections and mounted on a metal stub using double sided adhesive tape, sputter-coated with gold for 30 s under vacuum and observed under SEM (S-3400 N, Hitachi, Japan).

Amino groups of gelatin undergo crosslinking with –CHO groups of GTA to form a gel and the degree of cross-linking was determined by indirect method in which remaining free amino groups were calculated by 2,4,6-trinitro-benzenesulphonic acid (TNBS) assay (21) and the difference of the initially present free amino groups and remaining free amino groups was considered to be used in crosslinking. For this purpose, the composite graft samples were lyophilized and distilled water (500 µl) was added to

Table II Composition and Nomenclature of Composite Graft Containing Different Loadings of Gentamicin (Gen) and Weight Ratio of Porous Biomimetic Microcarrier Scaffolds (cPMS)

Nomenclature	Composition (%w/v) gentamicin (Gen) and biomimetic porous microcarrier scaffolds (cPMS)	
	Gen	cPMS
Gel-Gen (2%)-cPMS (0.1%)	2	0.1
Gel-Gen (2%)-cPMS (1%)	2	1
Gel-Gen (2%)-cPMS (5%)	2	5
Gel-Gen (5%)-cPMS (0.1%)	5	0.1
Gel-Gen (5%)-cPMS (1%)	5	1
Gel-Gen (5%)-cPMS (5%)	5	5

Fixed components: Aqueous gelatin solution (Gel; 15%w/v); Glutaraldehyde (GTA; 0.5%)

'Gen' refers to gentamicin and cPMS implies cytomodulin coupled porous PLGA microparticles

the preweighed samples (~10 mg) followed by probe sonication for 1 min at 60 amplitude. For TNBS assay, 4% sodium bicarbonate (500 µl) and 0.05% TNBS (500 µl) were added to the samples, incubated at 40°C for 2 h, and absorbance of the resulting solution was measured at 349 nm. A standard curve was plotted using gelatin (non-cross-linked) by treating various concentrations of gelatin with TNBS in a similar manner and degree of cross-linking was calculated using the equation 1.

$$\text{Degree of crosslinking}(\%) = \left\{1 - \left(\text{abs}_{\text{sample}}/\text{weight}_{\text{sample}}\right)/\left(\text{abs}_{\text{gelatin}}/\text{weight}_{\text{gelatin}}\right)\right\} \times 100 \quad (1)$$

Characterization of the Composite Skin Graft

The composite graft was characterized for fluid uptake ability, evaporative water loss (EWL), water vapor transmission rate (WVTR), elastic modulus and degradation which are the major requirements for any skin graft and play a vital role in efficient healing of wounds.

Fluid Uptake Study

Fluid absorbing capacity of the skin graft is an important parameter for maintaining a moist environment over the wound bed. For this study, composite graft was cast in the wells (1 ml/well) of a 24-well plate and crosslinked with GTA and gelation was allowed to occur for one hour. Discs were cut out from each well using a cork borer (Diameter 8 mm). These discs were dropped in vials containing PBS solution (pH 7.2) and incubated at 37°C to enable fluid uptake in the graft. At regular intervals of time, the weight of the graft was noted after removing excess PBS until equilibrium weight was reached and percentage fluid uptake was calculated using equation 2.

$$\text{Percentage fluid uptake} = (\text{Wt} - \text{Wo})/\text{W}_0 \times 100 \quad (2)$$

W_0 and W_t are initial weight and weight after time 't' respectively.

Evaporative Water Loss (EWL)

To measure the loss of water from the graft under dry conditions, sample discs were prepared as described above and incubated at 37°C and 32% RH. After regular intervals of time, the weight of the samples was noted and evaporative water loss was calculated using equation 3.

$$\text{Percentage weight remaining} = \text{W}_t/\text{W}_0 \times 100 \quad (3)$$

W_0 and W_t are initial weight and weight after time 't' respectively.

Water Vapor Transmission Rate (WVTR)

The moisture permeability of the composite graft was determined by measuring the water vapor transmission rate (WVTR) across the material. Sample discs of diameter 15 mm were prepared and mounted on the mouth of cylindrical plastic cups (14 mm diameter) containing 5 ml of water with negligible water vapor transmittance. The material was fastened using Teflon tape across the edges to prevent any unwanted water vapor loss through the boundary and kept at 37°C and 32% RH. The assembly was weighed at regular time intervals and weight loss *vs.* time plot was constructed. WVTR was calculated using following formula (equation 4),

$$\text{WVTR} = \frac{(\text{Slope} \times \text{A})}{24}\ \text{gm}/\text{m}^2/\text{day} \quad (4)$$

A is the area of the sample in m^2.

Mechanical Strength (Young's Modulus)

For testing of Young's modulus, samples discs were prepared (15 mm in diameter and 4 mm in height) and analyzed using texture analyzer (Stable Microsystems, TAXT2) equipped with a 5 kg-load cell. All samples were tested under "measure force in compression" mode using a test speed of 1 mm/min. Young's modulus was calculated from the slope of the linear portion of the stress–strain curve.

In Vitro Degradation Studies

Composite graft samples (1 gm) were freshly prepared and allowed to undergo crosslinking for 1 h. Crosslinked composite graft samples were incubated in PBS (100 mM, pH 7.4) at 37°C and 100 rpm in a reciprocal shaking water bath. At predetermined time intervals, PBS was removed and samples were lyophilized for the calculation of remaining weight (%) with respect to time. The morphology of degraded samples was also studied using SEM.

Evaluation of the Composite Graft

The composite graft was evaluated for *in vitro* drug release, cytocompatibility and antimicrobial activity.

In Vitro Drug Release Studies

In vitro drug release from the composite graft containing gentamicin at two different drug loadings (2% and 5%w/v) was carried out in PBS (100 mM, pH 7.4). In this study, samples were incubated in 2 ml of release media in a reciprocal shaker bath at 37°C and 100 rpm. At specific time

intervals, an aliquot of release media (1 ml) was withdrawn and replaced with an equivalent amount of fresh media. Drug content in the samples was analyzed by HPLC equipped with RF detector using pre-column derivatization of the drug with OPA in the presence of 2-mercaptoethanol using an *in-house* optimized method.

Cell Proliferation and Migration on the Composite Graft

Composite graft samples were formulated under aseptic conditions and allowed to undergo gelation for 1 h. Remaining free aldehyde groups of GTA if any, were quenched with a sterile aqueous solution of glycine (5%) and thereafter samples were washed twice with PBS. Human dermal fibroblasts (HDFs) were initially allowed to attach onto the wells of a 24-well plate. After cell attachment, sterile samples of composite graft were added into the wells and HDFs were allowed to grow in the presence of the graft samples. The cell growth and proliferation was evaluated by optical microscopy. In another experiment, HDFs were seeded directly onto the composite graft samples followed by the addition of growth medium (DMEM supplemented with 20% FBS and 25 mM HEPES buffer without any antibiotic drug) and incubated (37°C, 5% CO_2). Media was replaced on every 3rd day and cell proliferation was evaluated at day 10 by SEM.

Antimicrobial Activity Testing

Efficacy of the drug loaded composite graft to inhibit microbial growth was assessed by two methods, namely 1) Zone of inhibition (ZOI) measurement (Kirby- Bauer method) and 2) Time kill assay.

Zone of Inhibition Method (Kirby-Bauer Method). Sterilized media (nutrient broth containing 1.5%w/v agar) was added to the sterile culture dishes (90 mm) and allowed to set for 15–20 min. The bacterial broth suspension (equivalent to 0.5 McFarland standard) was spread uniformly onto the surface of the medium using a spreader (22). Inoculum was allowed to dry for 3–5 min and holes were bored into the plate using a sterilized cork borer (Diameter=8 mm). Sterile samples of composite graft were added into the holes. The plates were then incubated at 37°C and the zones of inhibition were measured daily.

Time Kill Assays. Time kill assays were conducted using a reported method (23). Briefly, sterile discs of composite graft were prepared using a cork borer (diameter 8 mm) and these discs were then added to the bacterial broth culture (1×10^6 CFU/ml), whereas untreated samples of bacterial broth were kept as growth control (n=3). Both samples were incubated at 37°C with constant agitation and bacterial broth (50 µl) was aliquoted from each sample at 0, 0.5, 2, 4, 24, 48 h, and plated after serial dilution to enumerate the number of surviving colonies following incubation for 24 h at 37°C. Counts were used to establish the number of Colony Forming Units (CFU) per ml of nutrient broth. Reinoculations of the bacterial broth culture of 1×10^6 CFU/ml were carried out at 24 h and 48 h.

Statistics

One-way ANOVA followed by Holm-Sidak test was applied for comparison among the different groups using Sigma Stats 3.5 for windows. Difference was considered statistically significant at level of $p<0.05$.

RESULTS AND DISCUSSION

Fabrication of Composite Skin Graft

Polymeric composite graft was prepared by using gelatin as a carrier gel for the biomimetic porous scaffolds (cPMS) along with antibacterial drug gentamicin. For the preparation of graft, gentamicin was dissolved in the aqueous solution of gelatin (15%w/v) to form a homogeneous solution and cPMS were dispersed in the same solution. A cross-linker GTA was added to the mixed suspension and gelation was allowed to occur for 1 h and composite skin graft was thus obtained. Cross linking in the system enhanced its mechanical strength and was mainly due to the formation of Schiff's base between the ε-amino groups or lysine and hydroxylysine side groups of gelatin and the aldehyde groups of GTA (24). It is assumed that part of the crosslinking was also due to free amino groups of gentamicin (structure shown in Table I) which also undergo crosslinking with GTA. Due to limitation of the TNBS assay method, separate crosslinking by GTA in gelatin and gentamicin could not be assessed, however total cross linking in the graft was evaluated by TNBS assay method and mechanical strength was also studied using texture analyzer. It was observed that the composite graft showed a degree of cross-linking of $99.36\pm0.27\%$ with an optimum mechanical strength as shown in later section. Surface morphology of prepared graft was assessed by SEM analysis of the freeze-dried sample which revealed that cPMS were uniformly distributed throughout the gelatin-gentamicin matrix (Fig. 1). Composite graft was prepared with 2% and 5%w/v drug loading and 0.1, 1 and 5%w/v cPMS were added to obtain six types of composite grafts (Table II). Percentage of scaffolds higher than 5%w/v of the composite graft resulted in a viscous fluid which did not form a strong gel even after cross linking with GTA.

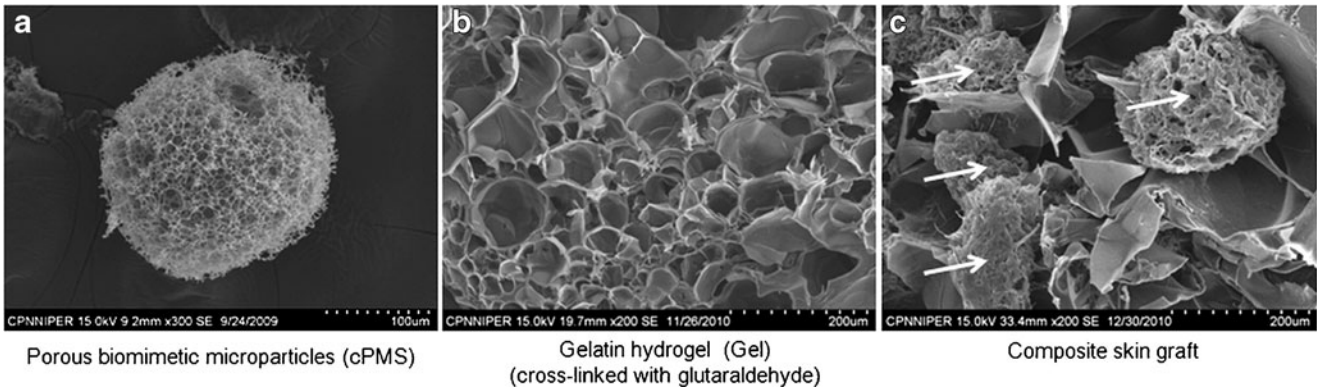


Fig. 1 SEM images of (**a**) cPMS (**b**) gel (**c**) composite skin graft. The composite graft sample shown here contains 2%w/v gentamicin and 1% cPMS in aqueous solution of gelatin (15%w/v) crosslinked with GTA (0.5%). Fragments of porous cPMS are clearly visible distributed uniformly in the gelatin gel (shown by *white arrows*).

This might be because of the physical barrier provided by microparticulate scaffolds at a higher percentage thereby reducing the crosslinking in the system. In addition to this, porous nature of the scaffolds also decreased the mechanical strength of the system (Fig. 2d) making it difficult to handle.

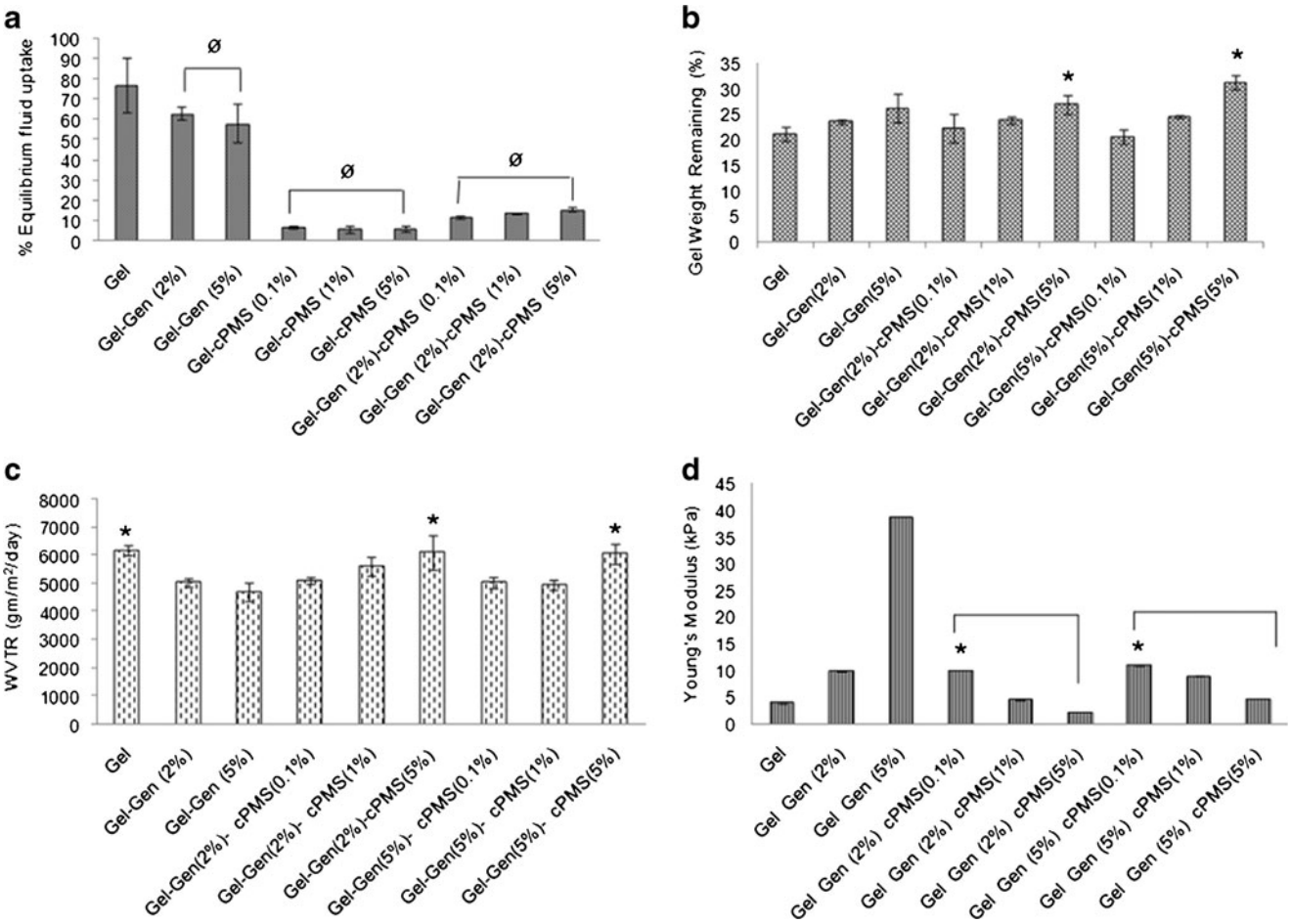


Fig. 2 Biologically relevant properties of composite skin graft in comparison with gelatin alone crosslinked with GTA (Gel) and gentamicin-gelatin matrix crosslinked with GTA (Gel_Gen). (**a**) % Equilibrium fluid uptake by composite skin graft; $P^{\varnothing} > 0.05$ represents no statistically significant difference among samples. (**b**) Evaporative weight loss (EWL); $P^{*} < 0.05$ *vs.* all other samples. (**c**) Water Vapor Transmission Rate (WVTR) $P^{*} < 0.05$ *vs.* all other samples. (**d**) Young's Modulus $P^{*} < 0.05$ *vs.* other samples. In all the experiments, data shows mean ($n=3$) ± S.D.

Characterization of Composite Skin Graft with Respect to Biologically Relevant Properties

The graft was optimized and characterized with respect to certain properties which are of biological significance in enhancing wound healing and include fluid uptake, evaporative water loss, water vapor transmission rate, mechanical strength and degradation of the graft.

Fluid Uptake Study

For any skin graft, fluid uptake by the graft is an important parameter because a wound generates fluid exudates which get accumulated at the site of injury and cause maceration and bacterial contamination. Thus, it becomes necessary that this fluid should be absorbed by the skin graft for faster and clean wound healing however; it should also not cause dehydration of the wound by excessive fluid absorption. This indicates that fluid uptake by the graft should be at an optimized level. It is reported that hydrogel based grafts show the advantage of limited fluid uptake due to their initial high fluid content (70–90%) (25) and this enable the hydrogels to maintain their integrity after imbibing wound fluid in addition to their inherent fluid content. Balakrishnan *et al.* reported an alginate dialdehyde and gelatin based synthetic hydrogel dressing which showed limited fluid uptake (5%) in addition to the initial fluid content of the gel (85%) while maintaining the integrity, a higher swelling or higher uptake could have resulted in loss of mechanical strength and integrity of the gel.

In the present study, carrier gel, gelatin (crosslinked with GTA) absorbed 77.02±13.51% of fluid in addition to the already present high amount of fluid in it (85%). Study was also performed to determine the fluid uptake capacity of the gel in the presence of the drug which showed a reduced fluid uptake of 60.80±3.42% as shown in (Fig. 2a). The reduction in the uptake was due to extra crosslinking of free amino groups of the drug molecules. This uptake was further reduced to 6.34±1.12% upon addition of cPMS to the gel (Fig. 2a). Addition of scaffolds caused a drastic reduction in the fluid uptake due to the hydrophobic nature of the microparticulate scaffolds. However, changing the weight ratio of the scaffolds did not change the fluid uptake significantly since the addition of more amount of cPMS increased the hydrophobic component of the system but also subsequently increased the system porosity. Graft samples containing 2%w/v gentamicin and different ratios of cPMS showed an average fluid uptake ~13.79±0.64%. It was also noticed that even after absorbing additional fluid, all the graft samples maintained their integrity during the study period.

Evaporative Water Loss (EWL)

EWL represents the loss of water from the composite graft when exposed to dry conditions (RH 32%) and was studied to examine its behavior under conditions applicable to dry wounds. Grafts based on hydrogel contain a large amount of water and thus show higher EWL. Commercially available product like Geliperm® (Geistlich Ltd., Switzerland) which contains 96% water, has been reported to show higher EWL of ~50% in 12 h, and retaining about 30% water after 24 h (24). In the present study, it was observed that in first few hours EWL was high (60–70% in 10 h) thereafter, no weight loss was seen even after 48 h and the samples retained 20–30% of their initial water content (Fig. 2b). EWL was minimum for the composite graft containing 5% w/w gentamicin in comparison to other test samples. This might be due to the additional cross-linking induced by the higher amount of drug present in the system. It was also noticed that graft samples containing higher amount of microparticulate scaffolds (cPMS) showed lesser EWL. This might be due to the hydrophobic nature of porous scaffolds which offers the hindrance in the system after the evaporation of water from the top layer of the graft samples.

These results indicate that the developed graft show the EWL in the range of 60–70% but keeping their top layer intact and may work more efficiently over moderately exuding wounds. The water loss from the composite graft would reduce the net water content of the graft and thus enable the graft to take up additional fluid from the exudates and edema of the wound by an upward directing process when used in an exuding wound.

Water Vapor Transmission Rate (WVTR)

Water vapor transmission rate is the rate at which the water is getting transmitted from the wound bed to the outside environment through the graft matrix. It is important that WVTR should not be very high for a healing wound to loss its integrity just due to dryness; on the other hand, a low WVTR increases the risk of wound maceration and contamination. Thus, an ideal wound graft must control the water loss from a wound bed to maintain a moist healing environment and avoid the presence of excess fluid at the wound site. WVTR of the currently available marketed products such as Geliperm® (Geistlich Ltd., Switzerland) and Vigilon® (Bard Ltd., Crawley, UK) shows the range of 9009±319 and 9360±34 g/m^2/day respectively (24). In the current study, WVTR through cross-linked gelatin (15%w/v aqueous solution) was found to be 6188.98±200.44 g/m^2/day (Fig. 2c) and 4881.95±233.85 g/m^2/day for cross-linked gelatin-gentamicin matrix. Further, addition of porous scaffolds to gelatin-gentamicin solution to fabricate composite skin graft increased the WVTR value proportional to the added amount of the

scaffolds and an average WVTR of all six types of grafts was found to be 5480.31±309.39 g/m^2/day.

High WVTR and high evaporative water loss would allow the composite graft to take up the fluids secreted at the wound site and prevent the underlying tissue from becoming over-hydrated and soft; thus, signifying that the graft is more suited for use in moderately/highly exuding wounds. For use in case of dry wounds, where a low WVTR is more desirable, the gel based composite graft could be covered with an occlusive dressing to reduce water vapor loss thereby the loss of fluids.

Measurement of Mechanical Strength

Skin graft should have the characteristic to ensure that it would maintain its integrity till the new skin completely replaces the damaged skin tissue with proper mechanical strength. In this study, carrier gel 1 h post-gelation with GTA showed a Young's modulus of 3.87 kPa which was increased to 9.80 kPa after addition of 2% gentamicin to gelatin gel and further increased to 37.3±0.05 kPa at 5% gentamicin as shown in Fig. 2d. This is due to the free amino groups of gentamicin which are playing part in additional crosslinking thereby increasing the mechanical strength. The same observation is also found in fluid uptake and EWL experiments. For composite graft, Young's modulus was found to be decreased as the percentage of microparticles (cPMS) was increased in the graft from 0.1% to 5% as shown in Fig. 2d. This might be attributed to the porous nature of the particles which reduces the mechanical strength of the graft.

These results indicated that EWL and WVTR at 0.1% scaffolds (cPMS) were low and very high at 5% cPMS. Moreover, 5%w/v cPMS also led to a significant decrease in the Young's modulus of the composite graft and also could not maintain the hydrogel property and thus, rejected. Additionally, 0.1% cPMS would have affected the cell adhesion and proliferation due to low quantity and thus, would result in inadequate skin regeneration. Thus, composite graft containing 1%w/v cPMS was selected for further experiments. Since there was no significant difference in the above properties at different drug loadings and drug was used only for combating local infection, both the drug levels within the graft were studied.

In Vitro Degradation

In the wound healing process, neotissue forms in due course of time which replaces the skin graft at the site of healing. Thus, biodegradability of the skin graft is important to provide an adequate space for skin regeneration. Considering the tendency of skin grafts to adhere to the wound bed, their biodegradability becomes even more important to circumvent the need of removal of the skin graft after healing has occurred, which might otherwise disturb the tissue undergoing healing process. In this study, composite graft consisted of components which are biodegradable in nature and their biodegradability was evaluated. For this purpose, samples were kept in PBS (100 mM, pH 7.4) for 4 weeks and their weight loss was measured. It was observed that carrier gel (gelatin crosslinked with GTA) lost 84.76±2.15% of the initial weight in 4 weeks of time and degraded at the fastest rate among all samples whereas, slow degradation was observed for gelatin-drug complex, Gel_Gen (2%) and Gel_Gen (5%) lost 45.99±3.40% and 51.10±1.17% weight respectively as shown in Fig 3a. Composite scaffold showed an intermediate degradation as weight loss for two tested samples Gel_Gen (2%)_cPMS (1%) and Gel_Gen (5%)_cPMS (1%) was 64.04±1.59% and 61.08±0.38% respectively. These results are on the same line as obtained for above discussed properties, where the gentamicin-gelatin complex showed highest Young's modulus due to formation of additional crosslinks with the drug molecule and thus justifying slowest degradation. Addition of porous cPMS to the fabricated graft reduced mechanical strength of the system and hence exhibited degradation faster than that of drug-gelatin complex. Gelatin being highly hydrophilic degraded most rapidly.

The same degradation pattern was also observed in SEM images (Fig. 3b) where carrier gel gelatin (Gel) losses its integrity and strength in 4 weeks of time. Gelatin-drug matrix showed an increase in porosity during the course of degradation, however, still maintained its structure after 4 weeks. Interestingly, in case of the composite scaffold, the gel matrix was completely degraded leaving apart only small fragments; though cPMS were still intact as seen in the figure inset (Fig. 3b) and maintained their porous nature. The combined degradation behavior of the composite graft was also correlated well with individual components of the composite graft and was serving the purpose for which it was designed. Gelatin merely acts as a carrier for both antibiotic drug and scaffolds and this system is expected to deliver the drug in a sustained manner and degrade after that; while the porous scaffolds should remain at the site for a longer time to support complete tissue regeneration.

Assessment of the Composite Graft

In Vitro Release Study

In wound healing, it is desired to have higher antibiotic drug concentration at the wound site to inhibit any possible infection from bacteria introduced during the initial trauma, followed by a prolonged sustained drug release at a therapeutically effective level to inhibit latent infection during the course of wound healing (4). In drug release

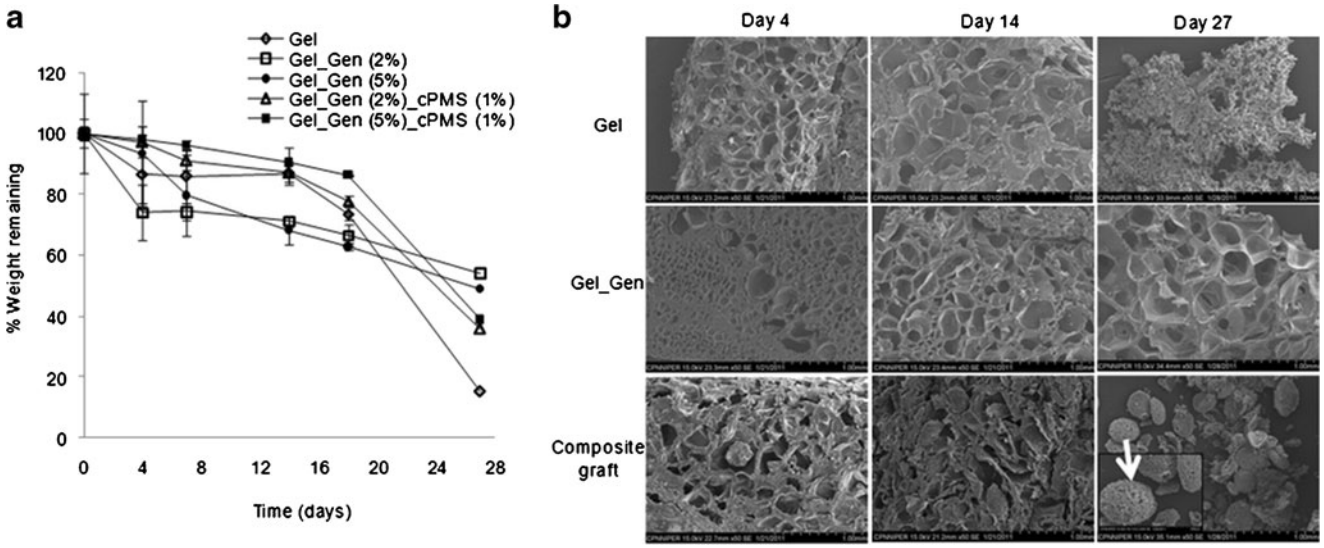


Fig. 3 *In vitro* degradation study of composite skin graft in comparison with gelatin alone crosslinked with GTA (Gel) and gentamicin-gelatin matrix crosslinked with GTA (Gel_Gen). (**a**) Weight change with time (data shows mean (n=3) ± S.D.), (**b**) SEM images showing the morphology of the degraded samples.

study, it was observed that the composite graft released 20–30% of the drug payload during the first day, which further increased to 50% by day 3 followed by a sustained and prolonged release for 13 days (Fig. 4). Gel_Gen (2%) _cPMS (1%) graft released 71.52 ± 2.98% of gentamicin while the drug release from Gel_Gen (5%)_cPMS (1%) graft was 56.10 ± 0.84% of the initial drug load for the tested period. Drug release from Gel_Gen (2%) and Gel_-Gen (5%) was 62.82 ± 1.54% and 61.23 ± 2.29% of initial drug loading respectively.

These results indicate that higher initial release would be effective to combat the microorganisms already present in the contaminated wound while a further slow and sustained release would suppress any new infections.

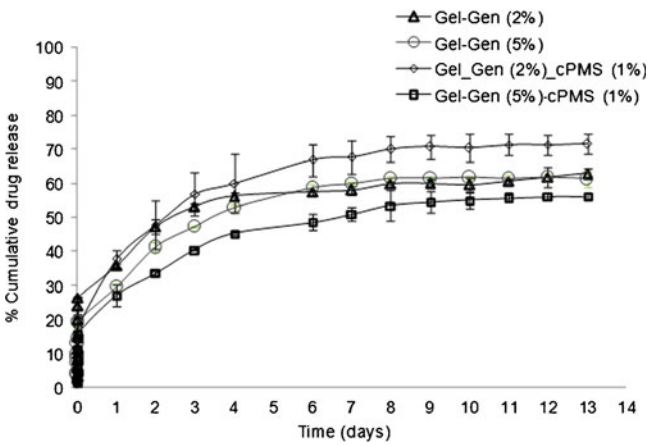


Fig. 4 *In vitro* drug release profile from the composite graft and gentamicin-gelatin matrix crosslinked with GTA (Gel_Gen) at drug loading of 2% and 5%w/v. Data shows mean (n=3) ± S.D.

Cytocompatibility

Cytocompatibility of the graft was evaluated by cell adhesion and proliferation on the graft, and was used to access the nature of cell-material interaction. To establish the cytocompatibility of the graft, HDFs were initially allowed to grow with the graft samples and cell growth was observed at day 1, 4 and 7. It was found that the cells were able to grow and proliferate in the presence of the graft. Interestingly, it was observed that cells migrated from the plate onto composite graft as depicted in Fig. 5a while maintaining their fibroblastic morphology and proliferated further on the graft till confluency was attained (Fig. 5b). This suggested normal behavior of the cells on the graft and hence the compatibility of material surface for cell growth and proliferation. The same behavior of the cells was observed on porous scaffolds (cPMS) in the composite graft, where cells were infiltrated into the porous scaffolds and proliferated further as shown in Fig. 5b. In another experiment, HDFs were directly seeded on the composite graft, allowed to grow and growth was observed by SEM as shown in Fig. 5c. SEM images revealed that cells adhered, grew and migrated over the graft; this indicates that the composite graft has the ability to support cell proliferation and migration.

Antibacterial Activity

Second major property which should be present in the composite graft, is to inhibit the growth of microorganisms. To evaluate this property, Kirby-Bauer and time kill assay methods were used using two microorganisms namely, *P. aeruginosa* and *S. aureus*. Both the microorganisms exhibited

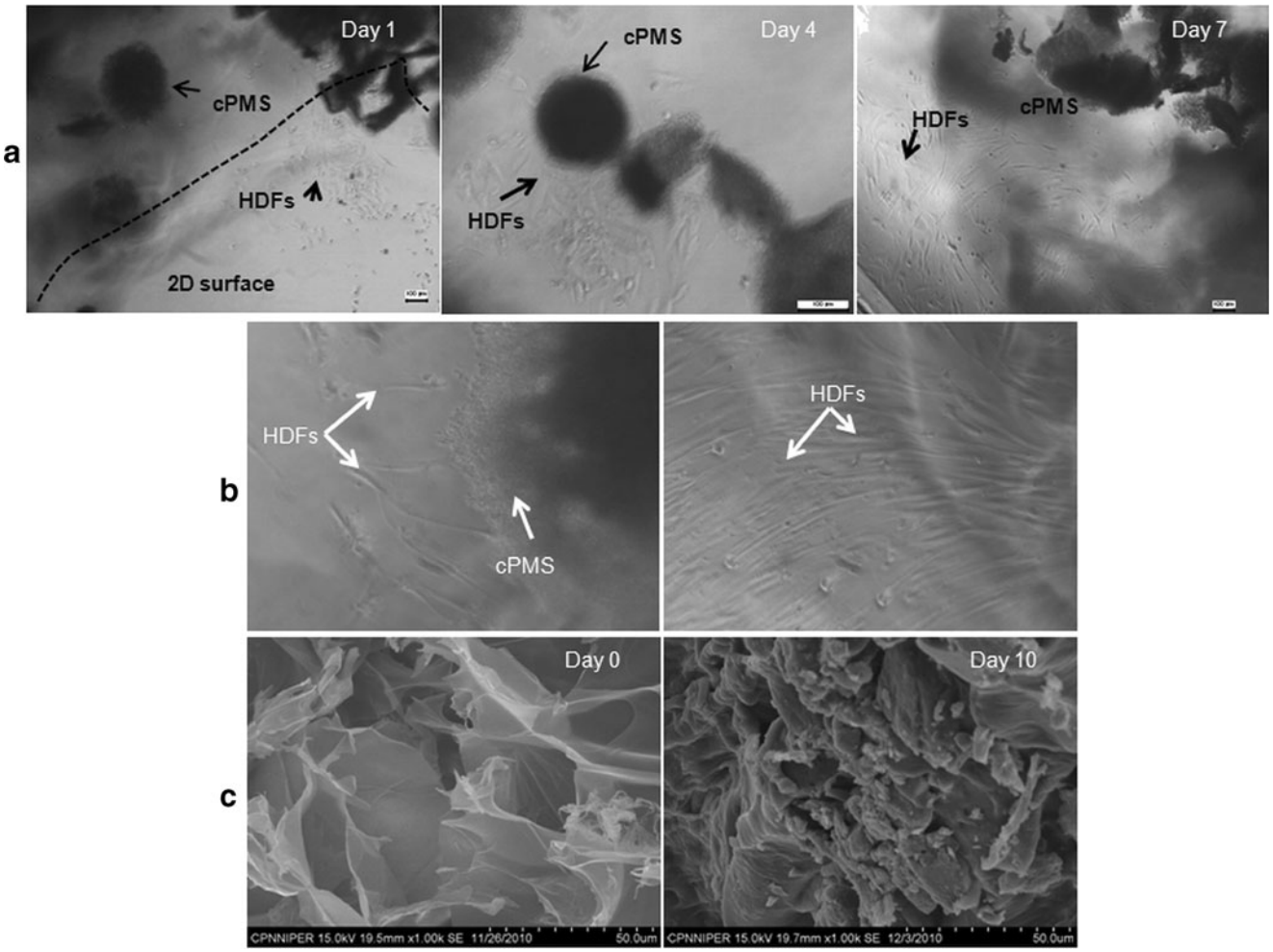


Fig. 5 *In vitro* cell culture images of HDFs cultured on the composite graft Gel_Gen (2%)_cPMS (1%). (**a**) Cell adhesion and proliferation (optical microscopy). (**b**) Cell migration from cPMS of the composite graft (day 7) and attaining confluency (day 10). (**c**) SEM images on day 0 and day 10.

growth in the nutrient agar culture dishes (blank) signifying that these organisms were in the active growth phase as seen in Fig. 6a. No zone of inhibition was observed in the culture plates containing gelatin cross-linked with GTA (no cPMS) and gelatin cPMS cross-linked with GTA (no drug) which was expected in these blank samples. This confirmed that neither gelatin nor the microparticles inhibited the growth of microbes. However, composite graft containing gentamicin showed zones of inhibition in the culture dishes of *P aeruginosa* as well as *S. aureus* which were further maintained for 15 days which indicated the effectiveness of the drug incorporated in the graft. Figure 6b shows the diameters of zones of inhibition obtained with different samples which were larger for higher drug loaded samples.

The samples of composite graft were further tested for their antimicrobial activity using time kill assays and examined for the rate at which an antibiotic agent killed bacterial isolates as shown by the log reduction assays (Fig. 6c and d). In normal course, a 3-log CFU/ml decrease in bacterial counts compared to the counts for growth control indicates an adequate bactericidal response. In case of *S. aureus,* a 6-log reduction was observed in 4 h. In case of *P. aeruginosa*, a 5-log reduction was observed with Gel_Gen (2%)_cPMS (1%) after 2 h, but 6–log reduction was found with Gel_Gen (5%)_cPMS (1%). Upon reinoculation of same concentration (1×10^6 CFU/ml) after 24 h, a 6-log reduction was observed in the growth of both the microorganisms. Reinoculation after 48 h also followed a similar trend. It is evident from antibacterial activity testing experiments that composite graft efficiently inhibited the growth of microorganisms for a prolonged duration of time and indicates that the graft would also be able to combat infection in the healing wound bed and thus enable infection-free healing without need of any external administration of antibiotic drugs.

CONCLUSIONS

In this study, an injectable biodegradable, drug-eluting composite graft is developed and characterized. The

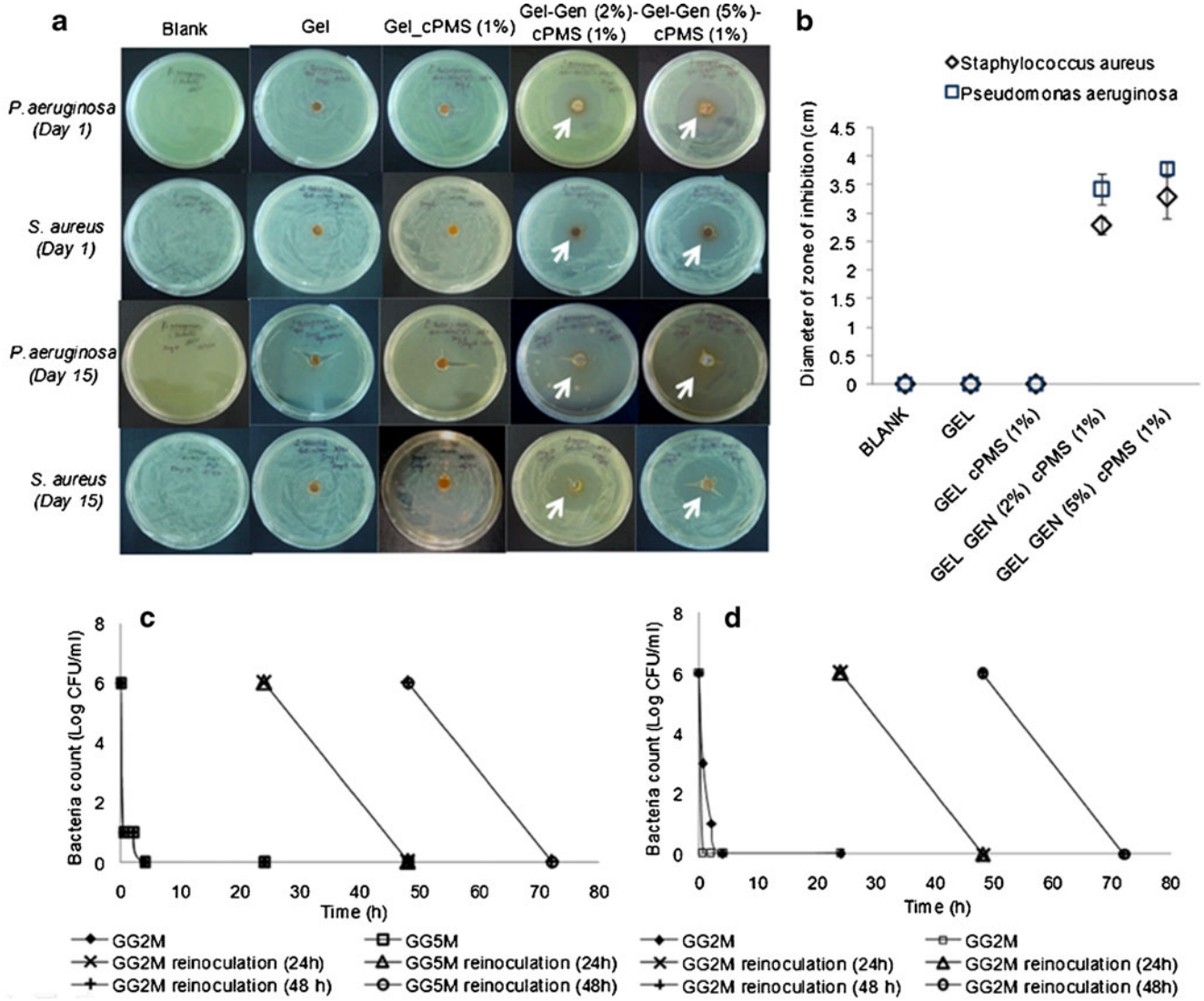


Fig. 6 Antimicrobial efficacy testing of the composite graft. (**a**) Visual observation of zones of inhibition formed by the composite graft. (**b**) Zone of inhibition measurement (data shows mean (n=3) ± S.D.). (**c**) Time kill assay for *S. aureus*. (**d**) Time kill assay for *P. aeruginosa* (data shows mean (n=4) ± S.D.). GG2M denotes Gel_Gen (2%)_cPMS (1%); GG5M denotes Gel_Gen (5%)_cPMS (1%).

developed graft is evaluated for the biologically relevant properties of a skin graft like fluid uptake, evaporated water loss, water vapor transmission rate and mechanical strength. It is observed that the developed graft has approached to the required criteria of these properties needed for a skin graft. Drug release studies suggested higher drug release at initial phase which became constant in the later phase which is highly desirable for inhibiting infection at early phase and to prevent the wound from further contamination. The composite graft also showed its potential for skin regeneration by supporting cell growth, proliferation and migration and also effectively inhibited the microbial growth. The study suggested successful development of a composite graft with respect to *in vitro* assessment which could be used for wound healing and to combat the microbial contamination simultaneously in the healing wound bed.

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