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Carboxymethylcellulose film for bacterial wound infection control and healing

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ABSTRACT

Infection control and wound healing profiles of sodium carboxymethylcellulose (SCMC) films were investigated as a function of their anti-bacterial action, physical structures, polymer molecular weights and carboxymethyl substitution degrees. The films were prepared with in vitro polymer/film and in vivo microbe-colonized wound healing/systemic infection profiles examined. Adhesive high carboxymethyl substituted SCMC films aided healing via attaching to microbes and removing them from wound. *Pseudomonas aeruginosa* was removed via encapsulating in gelling low molecular weight SCMC film, whereas *Staphylococcus aureus* was trapped in tight folds of high molecular weight SCMC film. Incomplete microbe removal from wound did not necessary translate to inability to heal as microbe remnant at wound induced fibroblast migration and aided tissue reconstruction. Using no film nonetheless will cause systemic blood infection. SCMC films negate infection and promote wound healing via specific polymer-microbe adhesion, and removal of *S. aureus* and *P. aeruginosa* requires films of different polymer characteristics.

1. Introduction

Sodium carboxymethylcellulose (SCMC) is ether cellulose characterized by carboxymethyl substitution. It is synthesised in molecular weights ranging between 90,000 and 2,000,000 g/mol, and has a degree of ether substitution varying from 0.6 to 1.0 (Sebert, Bourny, & Rollet, 1994; Kulicke et al., 1996; Sudhakar, Kuotsu, & Bandyopadhyay, 2006). The SCMC exhibits a high water bonding affinity with excellent skin and mucous membrane compatibility (Kulicke et al., 1996; Ludwig, 2005; Sudhakar et al., 2006; Liu, Liu, Wang, Du, & Chen, 2007). SCMC alone has been formulated as dressing for treatment of burn wound. It primarily maintains an optimal moist environment in wound region for extracellular matrix formation and re-epithelialization to take place (Ramli & Wong, 2011). The moist regulation of wound has been reported to have multiple advantages namely prevention of tissue dehydration and death, alleviation of pain sensation, promotion of angiogenesis, breakdown of dead tissue and fibrin, and interaction between growth factors and target cells (Field & Kerstein, 1994; Stashak, Farstvedt, & Othic, 2004).

A wound can be classified as open wound where the skin is torn, cut or punctured, or closed wound where blunt force trauma causes a contusion. The open wound is susceptible to bacterial colonization. The *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the common pathogens capable of causing clinically significant infection in colonized wound (Hutchinson & Lawrence, 1991; Field and Kerstein, 1994). A bacterial count greater than 10^5 colony forming units/g (cfu/g) of wound tissue can lead to infection. As such, the wound care of infected cases usually proceed with topical or systemic antibiotic treatment (Ljungh, Yanagisawa, & Wadström, 2006; Low, Martin, Hill, & Kenward, 2011). The topical antibiotic treatment is preferred over the systemic administration due to less widespread adverse effects. The *P. aeruginosa* and *S. aureus* can be resistant to topical antibiotic treatment through forming biofilm at wound, hindering the drug penetration and signalling to host immune system. Physical removal of the colonized bacteria is deemed necessary in wound care. It allows reduced bacterial colonies, facilitates antibiotic actions, lowers antibiotic doses and risks of antibiotic resistance.

The hydrosurgery has been introduced as a safe and effective method to reduce the bacterial load on wound bed without inflicting the viable tissue structure (Martin, Allan, Olson, & Nagel, 2011; Nusbaum et al., 2011). The hydrofiber made of carboxymethylated

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cellulose has also been demonstrated to be able to swell to form a cohesive gel for bacteria removal by encapsulation (Vloemans, Soesman, Kreis, & Middelkoop, 2001; Walker, Hobot, Newman, & Bowler, 2003; Newman, Walker, Hobot, & Bowler, 2006). Nonetheless, its bacteria removal characteristics at the wound–dressing interface and infection control capability have yet to be investigated with reference to the physicochemical attributes of polymers and dressings. The SCMC dressing has been reported to be able to promote healing of partial thickness wound through regulation of transepidermal water loss in a polymer molecular weight-dependent manner (Ramli and Wong, 2011). The wound healing however is minimally affected by the carboxymethyl substitution degree of polymer and its film nanoporous structure. This study aims to investigate the bacteria removal and infection control characteristics of SCMC films from wound surface as a function of polymer and dressing attributes, and their relation to healing of partial thickness wound with microbial burden. It is hypothesized that SCMC films prepared from polymers synthesizing with different molecular weights and carboxymethyl substitution degrees would have different physicochemical changes at microbe-burdened wound bed thereby removing bacteria by means of varying mechanisms and to different extents.

2. Materials and methods

2.1. Materials

Low molecular weight SCMC (LV, Sigma-Aldrich Chemie, Germany), medium molecular weight SCMC (MV, Sigma-Aldrich Chemie, Germany) and high molecular weight SCMC (HV, Sigma-Aldrich Chemie, Netherlands) were used to prepare polymeric films. *P. aeruginosa* (ATCC 27853) and *S. aureus* (ATCC 25923) were two bacterial species of interest (Microbiologics, USA). Other chemicals employed included sodium hydroxide (Merck, Germany) and disodium hydrogen orthophosphate anhydrous (Fischer Scientific, UK) for buffer preparation, sodium chloride (Merck, Germany) for wound cleansing solution preparation, Haeris haematoxylin (VWR International, UK), eosin (Microm International, Germany), xylene mixtures of isomers with dibutylftalan (VWR International, UK), hexane (Merck, Germany) and ethanol (Merck, Germany) for tissue staining preparation, glutaraldehyde (Electron Microscopy Sciences, UK), ethanol, hexamethyldisilazane (Electron Microscopy Sciences, UK) for scanning electron microscopy sample preparation, gentamicin and penicillin (Fisher Scientific, UK) as antibiotics in microbiological study, and ketamine hydrochloride and xylazine hydrochloride (Troy Laboratories, Australia) as anaesthesia.

2.2. SCMC film preparation

Two percent (w/w) LV suspension was hydrated for a minimum of 6 h at 25.0 ± 1.0 °C under magnetic stirring at 150 ± 1 rpm. Fifty gram of hydrated and air bubble-free SCMC solution were then transferred into a glass petri dish (internal diameter = 9 cm). The solution was subjected to hot air oven drying (Memmert, Germany) at 40.0 ± 0.5 °C for 5 days. The dried film was cut into circular matrices (diameter = 22 mm, average weight = 0.05 ± 0.01 g, average thickness = 0.127 ± 0.010 mm) by a stainless steel mould. These films were packed individually in a sealed heavy duty industrial plastic bag (Nu Kingston, Malaysia). They were sterilized through irradiating with gamma rays at a dose of 25 kGy (JS10000 Hanging Tote Irradiator, MINTec-Sinagama, MDS Nordion, Canada). The gamma rays-irradiated films were kept in desiccator until use. The same preparation process was applied to produce MV and HV films.

2.3. Animal wound induction

Healthy male *Sprague Dawley* rats (age = 3 months, body weight = 200 ± 20 g; Genetic Improvement and Farm Technologies Sdn Bhd, Malaysia) were acclimatized under 12 h light/dark cycle for 7 days in individual housing with deionized water and animal feed pellets provided ad libitum. The ambient temperature and relative humidity were kept at 25.0 ± 2.0 °C and 55.0 ± 2.0%, respectively, in automated caging system equipped with HEPA filter (Techniplast, Italy). The cages were cleaned and subjected to daily 70% (w/w) ethanolic solution swab.

At day 8, the animal was given intramuscular injections of ketamine and xylazine at 90 mg/kg and 10 mg/kg body weight of rodent, respectively. The hair at dorsal region was then removed by a sharp blade. The shaved area was cleansed with alcohol swab. The contact partial thickness wound was produced through placing 6 ml of deionized water heated to 65.0 ± 5.0 °C for 1 min in a circular plastic ring (external diameter = 2.20 cm; internal diameter = 1.27 cm) secured onto the shaved region of rat by an adhesive agent. Nine repetitive cycles were implemented on the same skin site with 1 min for each cycle. The wound area was swabbed by 70% (w/w) ethanolic solution and proceeded to wound healing study. All experiments were conducted according to institutional ethics policy adapting the international guidelines (OECD Environment, Health and Safety) (Organisation for Economic Co-operation and Development (OECD), 1994).

2.4. Bacterial inoculum preparation

Two pellets of *P. aeruginosa* (microbial content = 4.1×10^3 cfu/ml) or *S. aureus* (microbial content = 5.3×10^3 cfu/ml) were placed in 2 ml of hydrating fluid (Oxoid, UK) and left to incubate (INCUCELL, MMM Medcenter Einrichtungen GmbH, Germany) at 37 ± 1 °C for 30 min. The hydrated suspension was then homogenized (Vortex mixer, VELP Scientifica, Italy) and 1 ml of this dispersion was transferred to 9 ml of sterilized nutrient broth ('Lab-Lemco' powder, yeast extract, peptone and sodium chloride, Thermo Scientific, UK). The mixture was further homogenized and incubated for 24 h at 37 ± 1 °C. After 24 h, 10 ml suspension were diluted by 1 l of sterilized nutrient broth and subjected to incubation for an additional 24 h, followed by centrifugation for 10 min at 4000 rpm (Multispeed refrigerated centrifuge PK 121R, ALC International, Italy). The supernatant was removed and the bacterial sediment was transferred to a sterile container, cool to frozen at −20 °C for 2 h and dried using a freeze dryer (Eyela FDU 1200, Tokyo Rikakikai, Japan) at −45 °C and vacuum pressure of 14 Pa for 24 h. The dried bacterial sediment was kept refrigerated at −80 °C (Thermo Scientific, USA) until use. The bacterial inoculum required for wound healing study was prepared by suspending the sediment in USP phosphate buffer solution pH 7.2 with bacteria concentration adjusted to 10^6 colony forming units per 40 μl through bacteria plate counting technique using nutrient agar (yeast extract, triptone and glucose, Thermo Scientific, UK) as growth medium.

2.5. Wound healing study

The wounded rats were randomly divided into 12 per group as control, animals receiving LV, MV and HV films. In each rat, the wound was inoculated with 40 μl of freshly reconstituted bacterial suspension (10^6 cfu/40 μl) which consisted of either *P. aeruginosa* or *S. aureus*. After 2 min, a film was applied onto the wounded area with the aid of a standard 3 M adhesive tape except the control rats. The film was changed every 6 h for 48 h and there was no film applied onto the wound thereafter. The used films were subjected to scanning electron microscopic morphology and

bacterial count analysis. Similar to film, the excised wound tissue was subjected to bacterial count analysis. The macroscopic and histological morphologies of wound were examined at various intervals of treatment when needed.

2.6. Wound tissue morphology

The surface morphology of wound ($n = 5$/group) was examined by means of a digital camera (Olympus μ720 SW, Olympus, Japan) at various intervals of healing in the absence of film. The size of wound was measured using the digital micrometer (Mitutoyo, Japan) where the wound size was an average measurement from the longest and shortest dimensions of a wounded area.

2.7. Wound tissue histology

At a pre-determined period of healing, the rats ($n = 3$/group) were sacrificed. The wounded skin including its subcutaneous tissue was harvested for histological analysis. The skin specimens were frozen at −20 °C and fixed using Jung tissue freezing medium® (Leica, Germany). Vertical sections of skin with a thickness of 5 μm were produced from the fixed skin tissue using the cryostat (CM 1850 UV, Leica, Germany) and collected on glass slide. The microtomed samples were treated with xylene to remove the fat content, and subsequently cleaned and dehydrated by ethanol. The samples were then stained using haemoxylin and eosin system, and observed under a light microscope (ProgRes C3, Leica, Germany). At least 5 replicates were carried out on each wound area. The representative sections were photographed.

2.8. Bacteria-SCMC film interaction

The bacteria removal capacity of SCMC film was evaluated over 24 h of application via examining its adhesive interaction profile with bacteria using scanning electron microscopy technique, as well as, bacterial count available on film, wound tissue, blood and internal organs such as spleen, kidney, liver and lung. The examination of bacterial presence in blood and internal organs was essential to discriminate the chances of infection following bacterial colonization in wound and film application.

2.8.1. SCMC film morphology

The surface morphology of a film was examined using the field emission scanning electron microscopy technique (Quanta FEG 450, FEI, Holland). Owing to the presence of microbes (Walker et al., 2003), the film was subjected to fixation using 2.5% glutaraldehyde for 1 h followed by dehydration using aqueous ethanol solutions in the succession of 10, 25, 70, 90 and 100% when required. The film was then immersed in hexamethyldisilazane overnight and subsequently air-dried for 4 h. The dried film was coated with platinum and viewed directly under microscope at an accelerating voltage of 1 to 5 kV. At least triplicates were conducted and the representative sections were photographed.

2.8.2. SCMC film, wound tissue, blood and internal organ bacterial count

2.8.2.1. SCMC film. The film was removed and divided uniformly into three parts with the weight of each part recorded. The divided film was immersed in 50 ml of physiological saline with 0.1% (w/w) Tween 80. It was subjected to homogenization for 1 min at 25 °C (Vortex mixer, VELP Scientifica, Italy). A 100 μl of homogenate was sampled and diluted by 10 folds. A 100 μl of solution from this dilution was introduced onto a nutrient agar plate. The plate was incubated for 48 h at 37 ± 1 °C and bacterial colony forming units were counted by means of colony counter (Funke Gerber Labortechnik, Germany).

2.8.2.2. Wound tissue. The rats ($n = 4$/group) were sacrificed with tissue at the wound base removed and weighed. The tissue was then homogenized (Vortex mixer, VELP Scientifica, Italy) for 30 ± 5 min at 25 °C in 50 ml of physiological saline containing 0.1% (w/w) Tween 80. A 100 μl of homogenate was sampled and diluted by 10 folds. A 100 μl of solution from this dilution was introduced onto a nutrient agar plate. The plate was incubated and counted for bacterial colony forming units using the established protocol.

2.8.2.3. Blood. The rats ($n = 3$/group) had their blood collected. The orbital sinus area was first disinfected with 70% (w/w) ethanol solution. A 100 μl of blood were drawn using sterile heparinized capillary tube (Selzer Labortechnik, German) via orbital sinus and directly inoculated onto the nutrient agar plate for bacterial count after incubation using the established protocol.

2.8.2.4. Liver, lung, kidney and spleen. The rats ($n = 3$/group) were sacrificed with the internal organs collected. One hundred mg of each organ were extracted. The tissue was homogenized (Vortex mixer, VELP Scientifica, Italy) for 30 min in 9.9 ml of physiological saline containing 0.1% (w/w) Tween 80. Subsequently, 100 μl of the homogenate was sampled and diluted by 10 folds. A 100 μl of the suspension from this dilution was introduced onto a nutrient agar plate. The plate was incubated and counted for bacterial colony forming units using the established protocol.

2.9. SCMC film molecular weight

The molecular weight of SCMC film was assessed by gel permeation chromatography (1100 series, Agilent Technologies, Germany) equipped with a refractive index detector in accordance to the method developed by Ashikin, Wong, and Law (2010).

2.10. SCMC film degree of carboxymethyl substitution

An accurately weighed 2.5% (w/w) of sample, expressed with respect to potassium bromide (KBr) disc, was mixed with dry KBr (FTIR grade, Aldrich, Germany). The mixture was ground using an agate mortar into a fine powder. It was then compressed into a disc. Each disc was scanned over a wavenumber region between 400 and 4000 cm^{-1} at a resolution of 4 cm^{-1} using a FTIR spectrometer (Spectrum 100 FTIR system, Perkin Elmer, USA). The characteristic IR peaks were recorded. The carboxymethyl substitution degree of SCMC was calculated from the absorbance ratio at 1601.3–1625.3 cm^{-1} to 3262.2–3436.9 cm^{-1} of which depicting C═O of carboxymethyl moiety to O—H functional group of the main SCMC chain. A higher absorbance ratio value indicated a higher degree of carboxymethyl substitution degree. At least three replicates were carried out and the results averaged.

2.11. SCMC film bioadhesiveness

The bioadhesiveness of the film was determined using the texture analyzer (Stable Micro System Ltd, Surrey, UK) equipped with a 5 kg load cell. The intact rat skin was used as the substrate for ease of comparing the bioadhesiveness of films. The rat skin was isolated and stored at −80 °C. It was thawed to room temperature of 25 ± 2 °C prior to bioadhesiveness testing. The rat skin was characterized by average thickness, width and length of 0.68 ± 0.02 mm, 30.03 ± 0.03 mm and 30.09 ± 0.02 mm, respectively. It was first mounted onto the flat surface of the lower perspex using a double-sided adhesive tape positioned on the skin tissue away from the

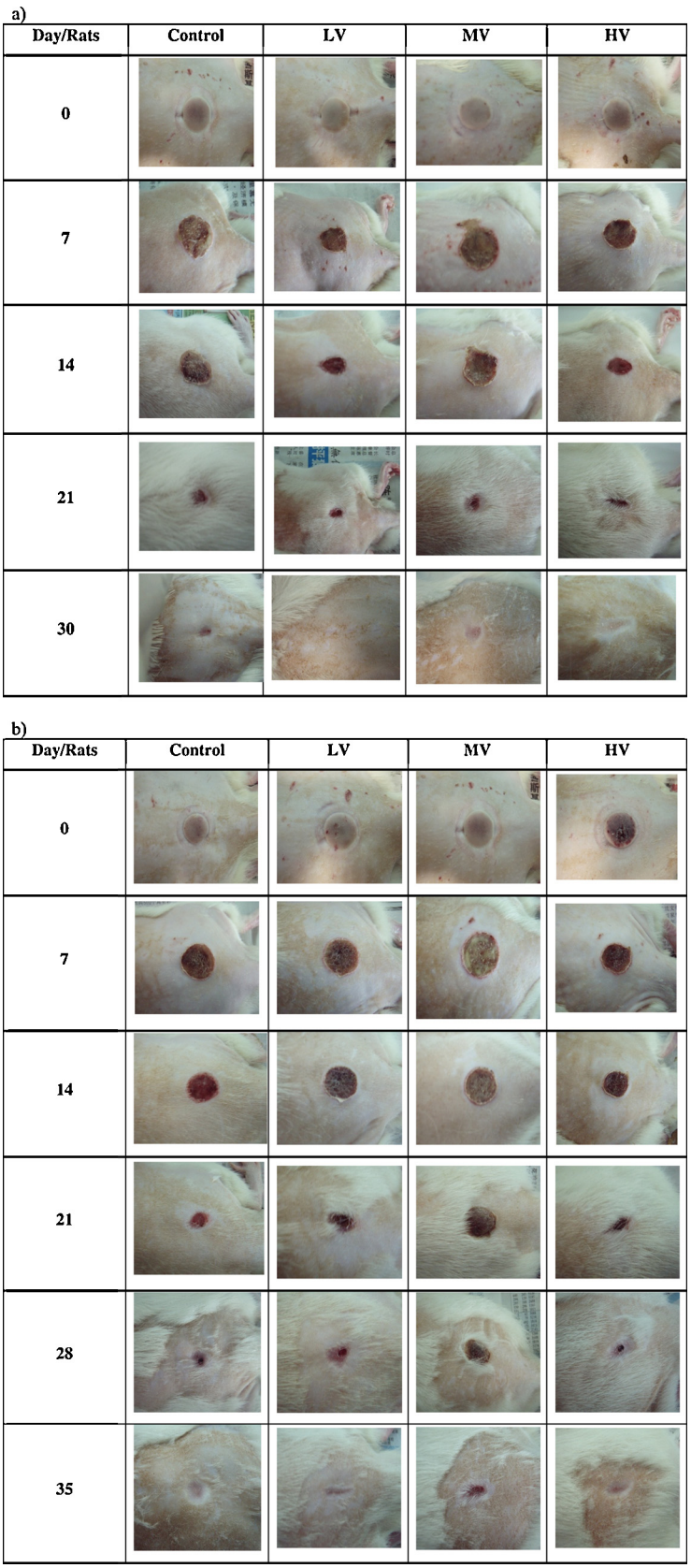

Fig. 1. Macroscopic wound profiles of rats inoculated with (a) *P. aeruginosa* and (b) *S. aureus* with no applied film (control) or treated by LV, MV and HV films.

surfaces of epidermis. The skin was further secured onto the perspex with peripheral fixation using aluminium foil and parafilm in order to avoid tissue displacement during the process of measurement.

A film, characterized by average thickness and diameter of 0.13 ± 0.01 mm and 2.20 ± 0.01 mm, respectively, was attached to the upper perspex using the double-sided adhesive tape. Both upper and lower perspex were aligned to ensure that the film came into direct contact with the top surfaces of rat skin. Prior to measurement, 100 μl of USP phosphate buffer pH 7.4 was introduced at the center region of the tissue surface. The upper perspex support was lowered with contacts established between the surface of the skin tissue and films for 180 s followed by its detachment at a speed of 0.5 mm/s. All measurements were conducted at 25 ± 1 °C and relative humidity of 55.0 ± 2.0%. The bioadhesiveness of a film was defined as peak detachment force required to separate the film from skin tissue. At least three replicates were conducted and the results averaged.

2.12. SCMC film microbial inhibitory effect

The microbial inhibitory test was used to examine the possibility of free polymer chains of film interacting with bacteria and inhibiting growth. 0.2 mg of dried bacterial sediment was diluted with 980 μl nutrient broth ('Lab-Lemco' powder, yeast extract, peptone and sodium chloride, Thermo Scientific, UK) and left in the incubator at 37 ± 2 °C for 30 min. The bacterial suspension was then sub-cultured by transferring 100 μl of the suspension into 3 ml of nutrient broth followed by further incubation for 24 h. After 24 h, the bacteria culture was streaked for a single colony and was transferred to 9.0 ml of nutrient broth for further growth with 24 h incubation. The microbial cell density of broth was read against the McFarland standard (standard number: R20412, Remel, USA) at the wavelength of 620 nm using the visible spectrophotometer (Thermo Electron, UK). Appropriate dilutions were made using nutrient broth, when required, to produce a broth with a cell density of 0.5 at wavelength 620 nm. Twenty μl of this broth was then streaked onto the nutrient agar plate and incubated for 24 h. The nutrient agar plate was used in disc diffusion test with positive control gentamicin (100 μM), negative control penicillin (100 μM), HV, MV and LV films ($n = 3$/disc) as the test samples. A larger inhibitory zone denoted a greater microbial inhibitory capacity of the samples. Solid films were used in disc diffusion test to emulate their actual physical state of application at the wound bed. The films were laid horizontally on the disc for 24 h at 37 ± 0.5 °C. The ability of solid film to inhibit the microbial growth would depend on interaction of

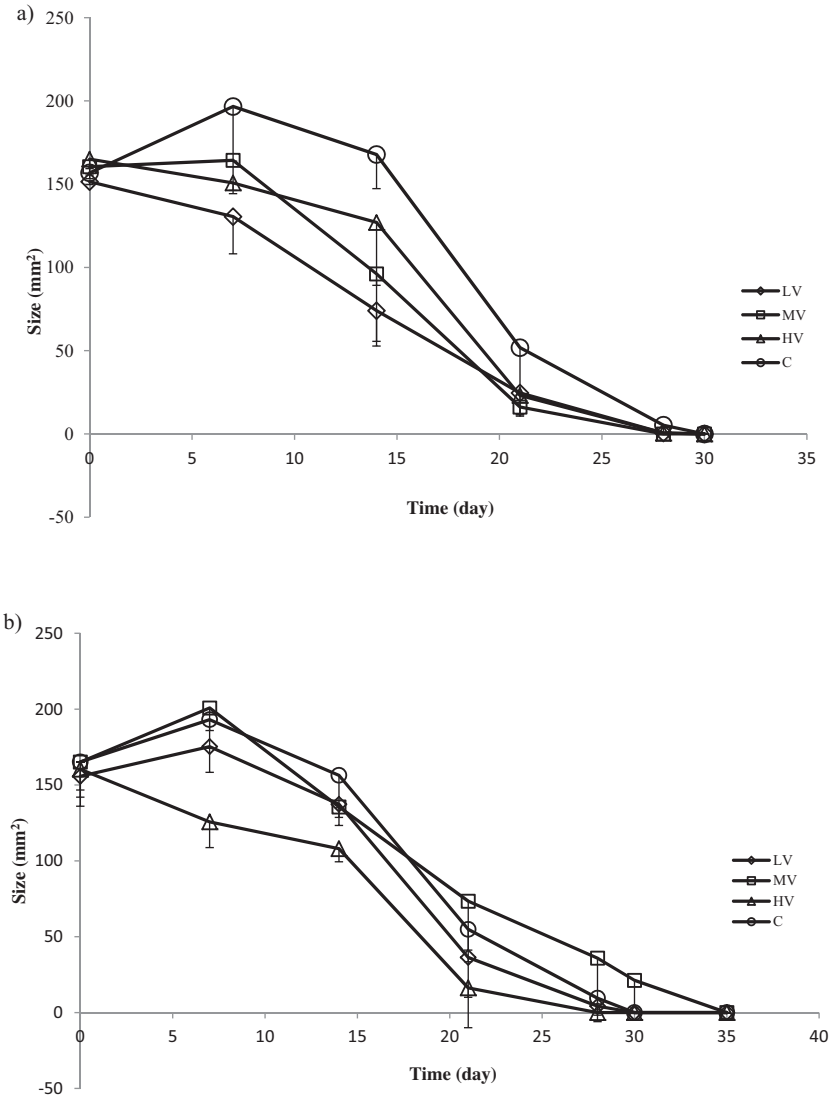

Fig. 2. Wound size profiles of rats inoculated with (a) *P. aeruginosa* and (b) *S. aureus* with no applied film (control) or treated by LV, MV and HV films.

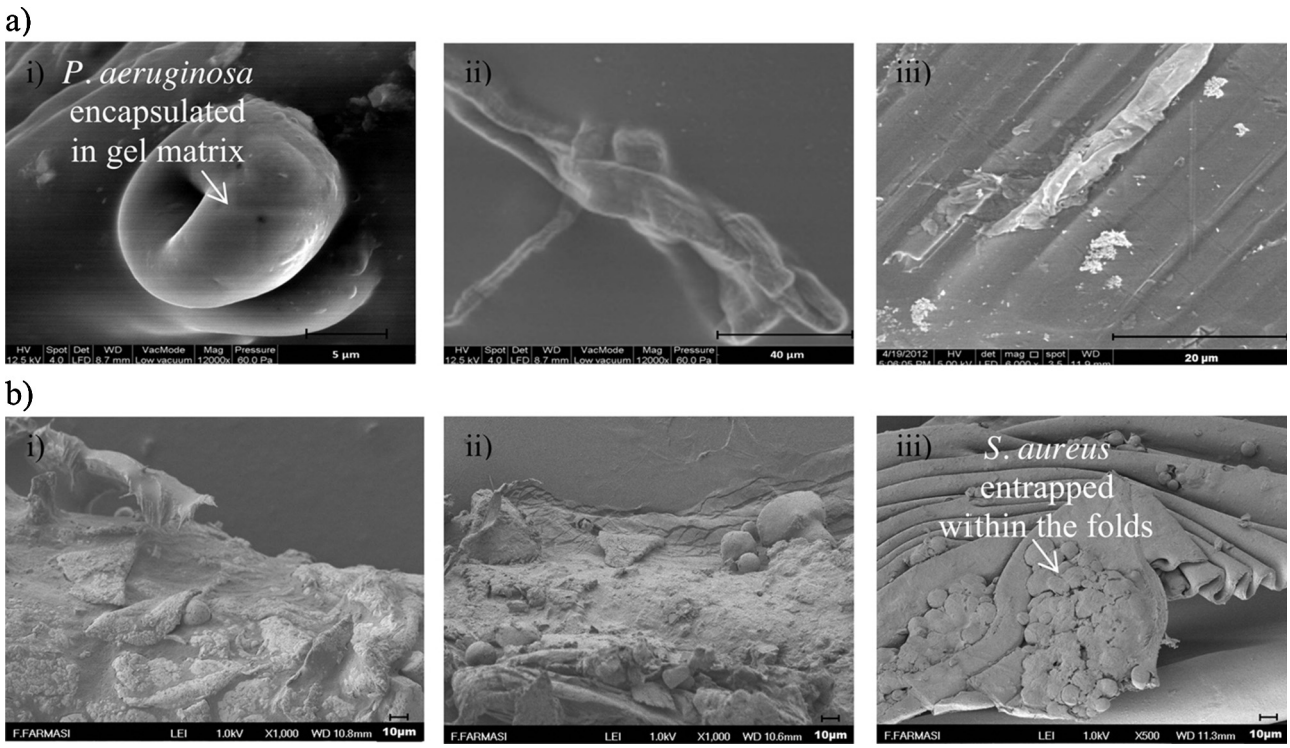

Fig. 3. SEM surface morphology of (i) LV, (ii) MV and (iii) HV films following their application on wound of rats inoculated with (a) *P. aeruginosa* and (b) *S. aureus.*

hydrated free polymer molecules with the bacteria. At least three replicates were conducted and results averaged.

2.13. Statistical analysis

All values were expressed in mean and standard deviation. Statistical data analysis was carried out using SPSS software version 19.0 and a statistically significant difference was indicated by $p < 0.05$. Two-way analysis of variance (ANOVA)/post hoc analysis by Tukey honestly significant difference (HSD) tests were employed to illustrate the differences in the wound size as a function of film type. The relationship of bioadhesiveness and polymer carboxymethyl substitution degree of SCMC films was examined using Pearson correlation analysis.

3. Results and discussion

The partial thickness burn wound was characterized by histology examination. It was marked by annihilation of epidermis and a large part of dermis as reported in the previous study of our laboratory (Ramli and Wong, 2011). Using no film, the wound loaded with *P. aeruginosa* and *S. aureus* required 28 to 30 days and 30 to 35 days, respectively, to complete its healing based upon the macroscopic observation and wound size measurement (Figs. 1 and 2). The wound was healed with a reduction in its circumference diameter with time from all directions of peripheral tissues (Fig. 1). The wound bed appeared to have a circular contour throughout the entire healing duration.

3.1. P. aeruginosa-colonized wound

The microbial-burdened wound exhibited a rise in size in the first 7 days of burn (Figs. 1a and 2a). The wound size reduced gradually between day 7 and 14, followed by a marked rate of reduction thereafter. The application of SCMC films was able to reduce the incidence of wound size expansion in the early healing phase (Fig. 2a; ANOVA: $p < 0.05$, day 0 against day 7). The films, either LV, MV or HV, aided to reduce the wound size throughout the entire period of healing (ANOVA: $p < 0.05$ against control). Among all, LV film demonstrated the best wound healing ability (ANOVA: $p < 0.05$ against control, MV and HV films).

All films were applied on the wound surface in the form of a solid state dressing. Inferring from disc diffusion tests, the LV, MV and HV films were not able to inhibit *P. aeruginosa* growth. These films, though hydrated in the aqueous milieu, could not be regarded as a chemotherapeutic agent (Table 1). Instead, the examination of used films from wound treatment indicated that the microbes were removed from wound through adhering onto the surfaces of films. It was the act of physical eradication of bacteria by films from wound bed contributed to healing. With reference to the scanning electron microscopy images, HV film was found to have a furrow structure where the microbes can possibly entrapped in its shallow grooves and be removed (Fig. 3aiii). The LV and MV films were characterized by a smoother surface morphology than HV film. Different from MV film, the LV film inclined to form gel structure on the wound surfaces possibly due to a greater ease of solvation by wound exudates. This was inferred by a prior study where lower molecular weight SCMC had a tendency to interact with moist more readily (Ramli and Wong, 2011). The LV film was envisaged to be able to encapsulate *P. aeruginosa* in gel matrix for removal from wound bed. The *P. aeruginosa* is known to secrete alginate as the extracellular product for biofilm formation and surface attachment (Tielen, Strathmann, Jaeger, Flemming, & Wingender, 2005). The formation of gel and bacteria encapsulation by LV film might be accompanied by viscous alginate secretion from the entrapped microbes. The bacterial alginate might act as the extra gelling material and result in the formation of strong SCMC gel structure for bacteria removal.

The strong bacteria removal capacity of LV film was supported by the microbial count of the used film and the wound tissue. The LV film demonstrated the highest count of microbes with the corresponding wound tissue characterized by the lowest level of microbe

Table 1
Physicochemical and microbiological characteristics of SCMC scaffolds.

Scaffold type	LV	MV	HV
Polymer molecular weight (g/mol)	$4.40 \times 10^4 \pm 4917.04$	$1.62 \times 10^5 \pm 5757.09$	$8.61 \times 10^4 \pm 1229.41$
Polymer carboxymethyl substitution degree	1.51 ± 0.84	1.28 ± 0.41	1.85 ± 0.68
Bioadhesiveness (mJ/cm^2)	210.97 ± 29.21	175.84 ± 11.65	324.14 ± 19.37
Zone of inhibition against *P. aeruginosa* and *S. aureus*[*]	Nil	Nil	Nil

[*] Zones of inhibition against *P. aeruginosa* and *S. aureus* for gentamicin are 10.0 ± 0.1 mm and 9.5 ± 0.1 mm, respectively; No marked zone of inhibition against *P. aeruginosa* and *S. aureus* exhibited by penicillin.

colonization (Fig. 4a). The MV film had neither furrow nor gelling structure (Fig. 3a). It on the other hand exhibited the lowest level of bacteria removal capacity (Fig. 4a). Numerous leucocytes were found to migrate to the wound bed treated by MV film at day 7 (Fig. 5). It was likely to be associated with the greater remnant of unremoved bacteria brought about by MV film, a phenomenon unfound in the cases of LV and HV films.

Analysis of SCMC molecular weight of films showed that MV film possessed the highest molecular weight SCMC (Table 1). The polymer molecular weight of MV film was unexpectedly higher than that of HV film, with reference to the gamma irradiation-free films where the polymer molecular weight increased in the order of LV < MV < HV (Ramli and Wong, 2011). This was due to ultra long polymer chains such as HV were more susceptible to breakdown under the irradiation of gamma rays during film sterilization (Miller & Shiedlin, 2002). Long MV polymer chains could translate to the formation of a more rigid film. Low levels of carboxymethyl substitution were accompanied by reduced ionized fractions essential for aqueous affinity. The MV film was constituted of SCMC of a lower carboxymethyl substitution degree than those of LV and HV films. This likewise led to the formation of film with a low gelling tendency. The summative effects could result in MV film expressing the lowest degree of bioadhesiveness (Table 1; Pearson correlation: Bioadhesiveness vs polymer carboxymethyl substitution degree, $r = 0.984$, $p = 0.056$), which aptly further explained its low bacteria removal capacity via physical adhesion and eradication processes.

In comparison to HV film, the low bacteria removal capacity of MV film did not seem to negate wound healing to a large extent except a slight tendency of wound to grow in size in the first 7 days of burn (Fig. 2a) and an excessive inflammatory response as

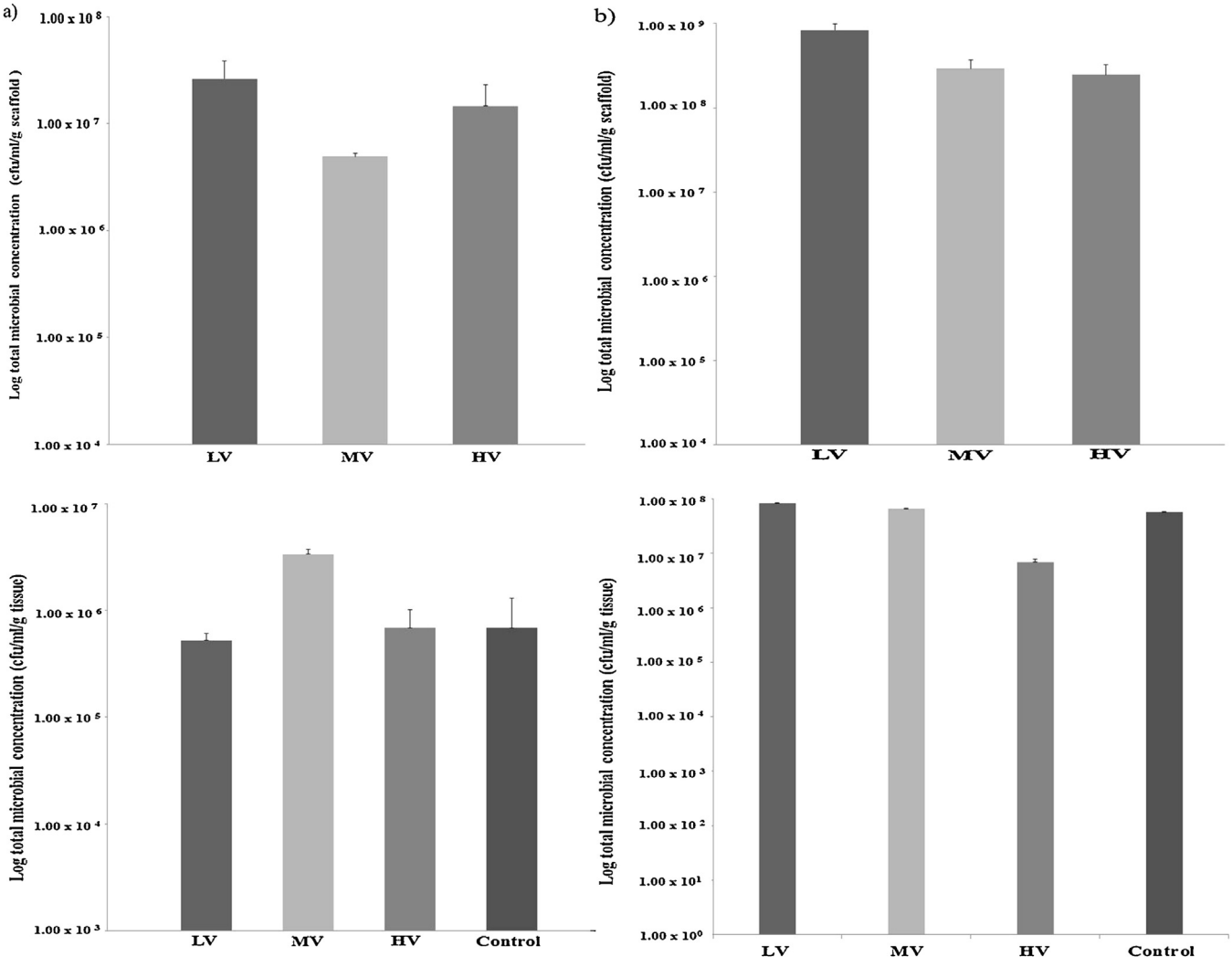

Fig. 4. Microbial count of film and wound tissue following film application on wound of rats inoculated with (a) *P. aeruginosa* and (b) *S. aureus*.

Table 2
Systemic infection profiles of rats with *P. aeruginosa* (*PA*) and *S. aureus* (*SA*) post 24 h inoculation of wound with microbes and treated with LV, MV, HV scaffolds or none.

	LV		MV		HV		Control	
	PA	*SA*	*PA*	*SA*	*PA*	*SA*	*PA*	*SA*
Blood	–	–	–	–	–	–	+	+
Spleen	–	–	–	–	–	–	–	–
Kidney	–	–	–	–	–	–	–	–
Liver	–	–	–	–	–	–	–	–
Lung	–	–	–	–	–	–	–	–

– Negative bacterial growth.
+ Positive bacterial growth.

previously described (Fig. 5). It was reported that reduction but not elimination of microbes could stimulate wound healing, probably through promoting fibroblasts and keratinocytes infiltration to the wound bed (Ljungh et al., 2006). Histological examination of wound tissue at day 14 following the treatment by MV film indicated that a marked entry of fibroblasts in addition to other cellular components took place (Fig. 5). The wound can be healed, though at a lower extent than that of LV film which exerted remarkable degrees of bacteria removal from wound bed.

Using no film, the control rats showed a surprisingly low microbial count at their wound tissue (Fig. 4a). Analysis of blood and internal organs indicated that the low microbial count could be a resultant effect of bacteria migration from colonized zone into blood when the film was not applied onto the wound bed (Table 2). The rats were infected by *P. aeruginosa* ($8.1667 \times 10^4 \pm 1.7677 \pm 10^4$ cfu/ml) when the films were not used to treat the wound. The microbes would only colonize at the wounded region instead of infecting the systemic circulation when the films were employed to remove the bacteria.

3.2. *S. aureus*-colonized wound

Similar to the case of *P. aeruginosa*, the LV, MV and HV films were not able to exert any inhibitory effect on the growth of *S. aureus* in disc diffusion tests (Table 1). However, their application on wound bed brought about a faster healing particularly when HV film was concerned (Figs. 1b and 2b; ANOVA: $p < 0.05$ against control, LV and MV films). The MV film, on the other hand, gave rise to a lower rate of wound size reduction in the late phase of healing than that of control with no applied film.

The HV film had the highest level of bioadhesiveness (Table 1). Its application resulted in a lower microbial count on wound bed (Fig. 4b). Nevertheless, the examination of the used HV film displayed an otherwise low level of microbe removal (Fig. 4b). The HV film creased into a form of corrugated folders upon placing on wound surface (Fig. 3b). Its effectiveness in microbe removal was not merely a function of matrix bioadhesiveness, but was also believed to be a consequence of microbe entrapment within the deep folds. The *S. aureus* appeared to be favorably entrapped in folds when compared to *P. aeruginosa*. This was probably related to its spherical coccus geometry allowing a greater extent of packing in the folds of film (Fig. 3biii). The microbial count on used film was an expression of viable colony possibly removed by the matrix. Unexpectedly low microbial count of used HV film might be ascribed to growth inhibitory effect in association with the compressive environment of folds (Denich, Beaudette, Lee, & Trevors, 2003). The *S. aureus* is a facultative anaerobic bacteria. Its growth in folds could be inhibited by compression force or acidity incurred

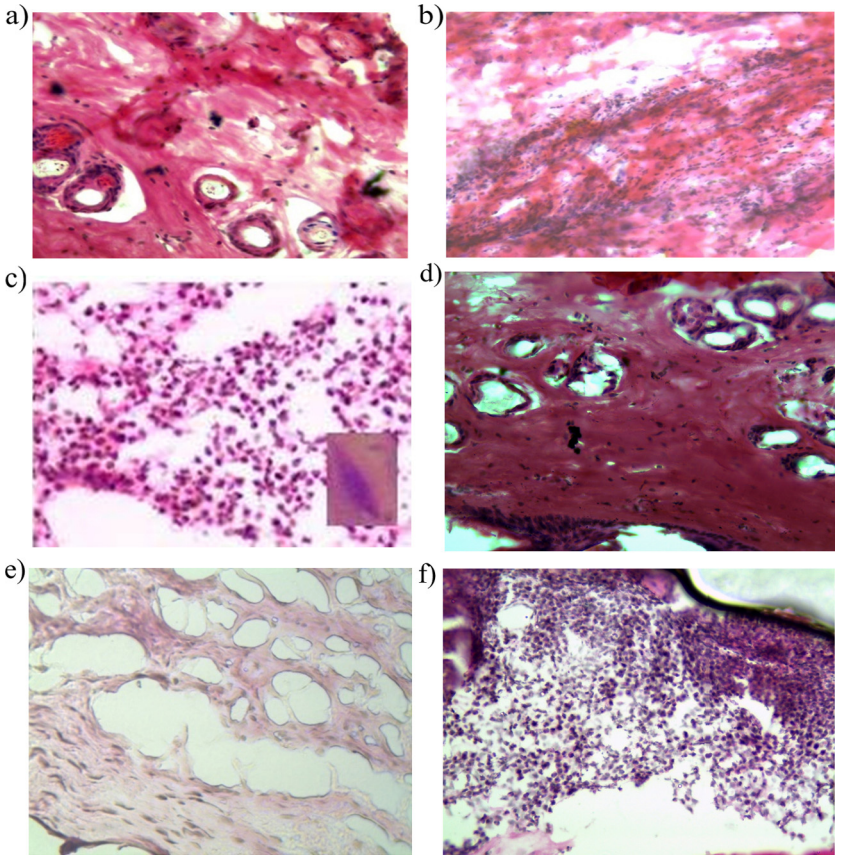

Fig. 5. Histology profiles of wound inoculated with *P. aeruginosa* and treated with MV film at (a) day 2, (b) day 7 and (c) day 14, and with *S. aureus* and treated with MV film at (d) day 2, (e) day 7 and (f) day 14 (inset in (c) was fibroblast).

by bacterial metabolites and/or waste products instead of oxygen tension. Preliminary trials conducted using dermal pH meter PH905 attached to a Cutometer 580MPA® (Courage + Khazaka, Germany) indicated that the pH values of skin and films were above 6. The growth inhibitory effect of HV film was not likely related to pH of the immediate environment of bacteria. It was most probably brought about by compression effect of folds.

Both film bioadhesiveness and physical structure had an essential role play in removal of *S. aureus*, unlike the case of *P. aeruginosa* where the ability of film to gel and consolidate the bacteria had a more dominant influence in microbe removal. Similar to the case of *P. aeruginosa*, the use of MV film was accompanied by an excessive inflammatory response due to low levels of microbe removal (Fig. 5). A dense presence of neutrophils could have destructive enzymes released and tissue matrix dissociation (Diegelmann, 2003). Using MV film to remove *S. aureus*, the propensity of oxidative stress brought about by inflammation reaction was greater than the associated repairing mechanism induced via fibroblasts and keratinocytes. This was clearly reflected by the extremely poor re-organization of extracellular matrix even at 14 days of healing when compared to the corresponding wound bed colonized with *P. aeruginosa*.

Using no film, the control rats had unexpectedly low microbial count at wound tissue (Fig. 4b). The bacteria had migrated from colonized zone into blood ($2.1667 \times 10^4 \pm 2.8868 \times 10^3$ cfu/ml) when the film was not applied onto the wound bed (Table 2). The rats were infected by *S. aureus* when the films were not used to remove the microbes physically from wound.

4. Conclusion

SCMC films are able to aid wound healing through early physical eradication of microbes from the tissue bed surfaces. The microbe removal ability of film is higher with an increase in its bioadhesiveness primarily due to high carboxymethyl substitution degree of the polymer chains. The bacterial removal characteristics and effectiveness of SCMC films differ with the type of microorganism. The *P. aeruginosa* can be effectively removed from wound through the gelling and encapsulation action of LV film. The removal of *S. aureus* is nonetheless effectively mediated via the ability of HV film to crease into multiple tight folders and accommodate the microbes in a compressive but microbial inhibitory environment. Incomplete removal of microbe from wound by film does not necessary translate to inability to heal. The remnant of *P. aeruginosa* at wound is found to induce fibroblast migration and aided tissue reconstruction. Using no film however will lead to systemic blood infection. The removal of *S. aureus* and *P. aeruginosa* requires films of different polymer characteristics. The success in identification of effective polymers and films is essential for subsequent pharmaceutical applications.

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