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Research paper

Biopolymer scaffolds for use in delivering antimicrobial sophorolipids to the acne-causing bacterium *Propionibacterium acnes*☆

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Sophorolipids (SLs) are known to possess antimicrobial properties towards many species (particularly Gram-positive, or Gram$^+$) of bacteria. However, these properties can only be exerted if the SLs can be introduced to the bacterial cells in an acceptable manner. *Propionibacterium acnes* is the common bacterial cause of acne. It is a Gram$^+$ facultative anaerobe that is susceptible to the antimicrobial effects of SLs. In this study we demonstrated that different biopolymer matrices could be used to produce SL composite films that exert various antimicrobial efficiencies against *P. acnes*. Increasing SL concentrations in poly-3-hydroxybutyrate (PHB) and PHB-*co*-10%-3-hydroxyhexanoate (PHB/HHx) resulted in noticeably improved (PHB/HHx was best) antimicrobial activity based on the size of the zones of inhibition using an overlay plating technique on synthetic growth medium. However, increasing concentrations of SLs in PHB and PHB/HHx films also increased film opacity, which diminishes the appeal for use especially in visible (facial) areas. Pectin and alginate improved the transparent character of SL composite films while also acting as successful carriers of SLs to *P. acnes*. The lactone form of the SLs proved to exhibit the best antimicrobial action and in concert with either pectin or alginate biopolymers provided a comparatively transparent, successful means of utilizing SLs as a renewable, environmentally benign anti-acne solution.

Article Outline

Results and discussion

References

Introduction

Acne vulgaris (simply known as acne) is a common skin condition that occurs most often during the teenage years. Aside from scarring, its primary effects are generally psychological, such as reduced self-esteem [1] and may, in some instances lead to more serious conditions of depression and/or suicide [2] and [3]. In 2006 in the United States alone, 45 million people were affected by various forms of acne and even now, $100 million per year is spent on over the counter remedies [1]. Since acne typically appears at a point in life when people already tend to be most socially insecure, timely treatments are advocated to reduce the overall impact to individuals [1].

Acne develops as a result of follicular blockage. Under normal circumstances, the cells lining the inside of a hair follicle slough off (desquamate) and are then forced out of the follicle by the growing hair. However, this process can be interrupted and some of these dead skin cells may remain in the follicle due to the presence of excess keratin, a natural protein found in the skin, which results in an increased adherence/bonding of dead skin cells together. This cohesion of cells blocks the hair follicle which, when paired with the naturally occurring sebum, results in ideal conditions for the formation of acne. When a pore is blocked, *P. acnes*, a slow growing, facultatively anaerobic, $Gram^+$ bacterium that lives on fatty acids in the sebaceous glands in a largely commensal relationship as part of the normal skin flora, overgrows and secretes chemicals that break down the wall of the pore, providing a favorable environment for the colonization of additional normal flora bacteria such as *Staphylococcus aureus*, ultimately resulting in the formation of acne lesions (folliculitis [4]).

There are many products available for the treatment of acne. In many cases, the modes of action are not fully understood but in general treatments are believed to work in at least 4 different ways (with many of the best treatments providing multiple simultaneous effects): (1) controlling shedding into the pore to prevent blockage, (2) killing *P. acnes*, (3) anti-inflammatory effects, and (4) hormonal manipulation. A combination of treatments can greatly reduce the amount and severity of acne in many cases. Those current treatments that are most effective tend to have greater potential for side effects and need a greater degree of monitoring, so a step-wise approach is often taken. There are several treatments that have been proven effective however; each effective method has its limitations. Topical bacteriocidal products, typically containing benzoyl peroxide may be used in mild to moderate acne. In addition to its therapeutic effect as a keratolytic (a chemical that dissolves the keratin plugging the pores) benzoyl peroxide also prevents new lesions by killing *P. acnes* [5]. Benzoyl peroxide has the advantage of being a strong oxidizer (a mild bleach) and thus, unlike antibiotics, does not lead to bacterial resistance. However, it routinely results in dryness, local irritation and redness and can very easily bleach any fabric or hair it comes in contact with. Antibiotics have also been used either through topical or oral applications to help treat acne. Erythromycin, clindamycin or the tetracycline antibiotics kill *P. acnes* but do nothing to reduce the oil secretion and abnormal cell behavior that is the initial cause of the blocked follicle. Additionally the antibiotics are becoming less and less useful as resistant strains of *P. acnes* are becoming more common, and the acne will generally reappear relatively soon after the end of treatment, days later in the case of topical applications, and weeks later in the case of oral antibiotics. Other less well-established procedures include the use of hormonal treatments (cortisone), topical or oral retinoid use (vitamin A-related compounds [6]) and phototherapy [7], [8] and [9].

Microbial glycolipids have also been demonstrated to be effective bacteriocidal agents against many different bacterial species. It has been reported that specific glycolipid biosurfactants show growth inhibition towards certain microorganisms because of their high surface activities, which lead to cell membrane damage [10]. In fact, research has shown, that although not a hard and fast rule, glycolipids tend to be more effective against $Gram^+$ bacterial strains than $Gram^-$ strains because of the structural variation between $Gram^+$ and $Gram^-$ cell envelopes [11]. This is certainly true for mannosylerythritol lipids (MEL) which have shown a high antimicrobial activity particularly against $Gram^+$ bacteria and tend to have minimum inhibitory concentrations (MIC) that are much smaller than those of sucrose monodecanoate and sorbitan monododecanoate (Span 20 [12]). Sophorolipids (SLs), sugar-based surfactants, have been shown to be biologically active compounds. They are composed of a disaccharide (sophorose) and a hydrophobic fatty acid tail (generally 16 or 18 carbons in length) that may be lactonized (preferred) to the 4″ position of the sophorose or remain in the open-chain form. In addition, SLs may be non-, mono- or diacetylated at the 6′ and/or 6″ positions on the sophorose sugar (for chemical structures of SLs see Fig. 1). It has been determined that certain structural analogues of SLs can inhibit the germination of conidia of the fungus *Glomerella cingulata* [13] as well as act as spermicidal and anti-HIV virucidal agents [14] in addition to inhibiting the growth of some $Gram^+$ bacteria including *Bacillus subtilis*, *Micrococcus luteus* and *Mycobacterium rubrum* [11]. Interestingly, in its lactone form SLs have also been shown to inhibit the growth of some $Gram^-$ bacteria including *Escherichia coli*, and *Serratia marcescens* [11]. Because of this known antimicrobial activity towards many bacterial species and because of its nontoxic nature towards humans in topical applications, we set out to determine the effects of SLs on *P. acnes* using various naturally derived polymeric matrices including poly-3-hydroxybutyrate (PHB), PHB-*co*-3-hydroxyhexanoate (PHB/HHx; NODAX™), pectin, and alginate (for chemical structures see Fig. 2) with the idea that use of SLs in anti-acne applications may result in an effective treatment without the potential side-effects that are currently present in other treatments while at the same time providing an additional outlet for SLs and biopolymers in a potentially large niche market.

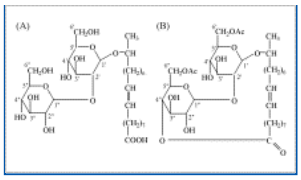

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Figure 1.

Chemical structures of 17-L-[(2′-*O*-β-glucopyranosyl-β-D-glucopyranosyl)-oxy]-9-octadecenoic acid sophorolipids in the nonacetylated, free-acid (open chain) form **(a)** and 17-L-[(2′-*O*-β-glucopyranosyl-β-D-glucopyranosyl)-oxy]-9-octadecenoic acid 6′,6″-diacetate sophorolipids in the 1′,4″-lactone form **(b)**.

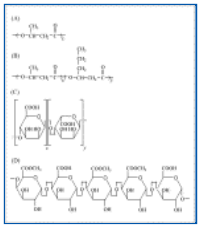

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Figure 2.

Chemical structures of poly(3-hydroxybutyrate) (PHB **(a)**), PHB-*co*-10%-3-hydroxyhexanoate (PHB/HHx **(b)**), alginate (copolymer of β-D-mannuronic acid and α-L-guluronic acid **(c)**), and pectin (copolymer of α-galacturonic acid and α-galacturonic acid methyl esters **(d)**).

Materials and methods

Materials

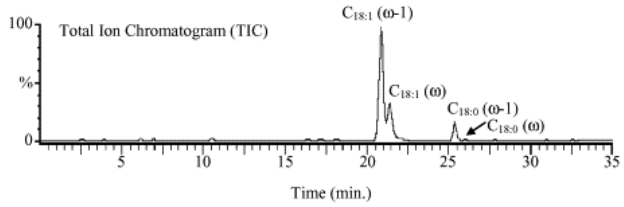
All media components involved in SL and PHB synthesis were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO) with the exception of Bacto™ yeast extract and Bacto™ tryptone which were purchased from Becton Dickinson Company (Sparks, MD). Pectin (low molecular weight), alginate and glycerol were purchased from Sigma–Aldrich, and hyaluronate was supplied by Lifecore (Chaska, MN). *Candida bombicola* ATCC 22214 was purchased from the American Type Culture Collection (Manassas, VA), while *Pseudomonas oleovorans* NRRL B-14682 and *P. acnes* NRRL B-4224 were obtained from the ARS Culture Collection (National Center for Agricultural Utilization Research; NCAUR, Peoria, IL). All microorganisms were stored at −80°C in their respective culture medium supplemented with 15% (v/v) glycerol as a cryopreservative. Poly-3-hydroxybutyrate-*co*-10%-3-hydroxyhexanoate (PHB/HHx; NODAX™) was kindly provided by Dr. Phil Green of Procter & Gamble (Cincinnati, OH). All solvents were HPLC grade and purchased from J.T. Baker (Phillipsburg, NJ).

Sophorolipid synthesis, isolation, purification and analysis

C. bombicola ATCC 22214 was used to synthesize the SLs used in this study. The inoculum was prepared in Candida Growth Medium (CGM), which consisted of the following in g/L: glucose, 100; yeast extract, 10; urea, 1, according to a previously published method [15]. Sophorolipid production was performed at the 10 L scale using a fed-batch fermentation protocol in a New Brunswick Scientific Bioflo 3000 bench-top fermenter (Edison, NJ). The fermentation media (CGM) was composed as described above with a starting pH of 6.0. The media were autoclaved to sterilize and then the temperature equilibrated to 26°C and oleic acid was added as the lipid co-substrate at a final concentration of 2% (w/v). A 50 mL frozen inoculum was thawed and used to inoculate the fermentation. The fermentation conditions were as follows: temperature = 26°C, agitation (impeller speed) = 700 rpm, air flow = 2 L air/min with no pH control. After 2 days, an additional 7.5% (w/v) dry glucose and 2% (w/v) oleic acid were added to the fermentation. At 5 days post-inoculation 1% (w/v) oleic acid was added and at 6 days an additional 0.5% (w/v) oleic acid was added and the fermentation allowed to proceed to completion for 1 additional day (total duration of the fermentation was 7 days).

Sophorolipids were isolated by first, lyophilizing the entire culture (cells and broth) to dryness (~2 days at −55°C and 30 mtorr) and then dividing and placing the dried residue into 4 separate 4 L Erlenmeyer flasks. Each portion was extracted with excess ethyl acetate by agitation on a magnetic stir plate at room temperature. The extracts were filtered through Whatman No. 2 filter paper and the remaining solids were washed twice more (3 extractions total) with ethyl acetate (1 L each time) to maximize recovery. The combined ethyl acetate fractions containing the SLs were concentrated by evaporation and added to 2 L of hexane to precipitate the pure SLs. The pure SLs were recovered from the hexane by filtration and vacuum dried in a desiccator to obtain a fine white powder and analyzed by liquid chromatography/mass spectrometry (LC/MS) as described previously [16] to determine the absolute content of the SL mixture (for content see Table 1).

Table 1. Conformation (open-chain vs. lactone) and the lactone distribution (*incl.* fatty acid profile and linkage data) for sophorolipids produced from glucose and oleic acid based on LC/MS analysis.



Sample	Open chain (%)[a]	Lactone (%)[a]	Lactone distribution (%)			
			$C_{18:1}$		$C_{18:0}$	
			ω−1	ω	ω−1	ω
SL-oleic acid	2	98	68	24	7	1

[a] Open chain sophorolipids eluted within the first 15 min while lactonic sophorolipids eluted after 15 min.

The open-chain form of SL was prepared by basic hydrolysis of the lactonic form. Briefly, SL (5 g) was dissolved in a solution of 1.5 g KOH in 30 mL water and stirred at room temperature (RT) overnight. The reaction was then neutralized with 6 M HCl to a pH of approximately 2, poured into a crystallizing dish, and the solvent was removed under a stream of nitrogen. The sticky residue was then mixed well with 50 mL of 2:1 $CHCl_3$/MeOH (v/v) and filtered through a pad of celite with sand on top to remove most of the salts. Solvent was removed; the residue was dissolved in a minimum amount of 9:1 $CHCl_3$/MeOH (v/v) and passed through a short column of silica gel. Elution with that solvent removed any remaining acetic acid (produced by hydrolysis of the acetate from the SL 6′ and 6″ hydroxy groups, see Fig. 1a), and the desired open-chain SL was then eluted from the silica with 70:30:2 $CHCl_3$/MeOH/H_2O (v/v/v). Thin-layer-chromatography (TLC) analysis (elution solvent: 80:20:1 $CHCl_3$/MeOH/H_2O (v/v/v); visualization by charring with a 5% phosphomolybdic acid solution in ethanol) showed no lactonic SL (R_f = 0.8), only the open-chain free acid form (R_f = 0.15) was present. This material was used without further purification.

Poly(3-hydroxybutyrate) synthesis, isolation and purification

P. oleovorans NRRL B-14682 was used to synthesize the PHB used in this study. The bacterial inoculum was prepared by aseptically adding 1 mL of culture from a frozen cryovial into a 125 mL Erlenmeyer flask containing 50 mL of sterile Luria-Bertani (LB) broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl) and incubating the flask at 30°C with rotary shaking at 250 rpm for 24 h. Then, the entire 50 mL culture was aseptically added to a 2 L Erlenmeyer flask containing 1 L of LB broth and the culture incubated for an additional 24 h as described above. The actual PHB production was performed at the 10 L scale using a batch fermentation protocol in a New Brunswick Scientific Bioflo 3000 bench-top fermenter containing medium *E** (pH 7.0) (for medium composition see [17]) and 2% glycerol (99.9% pure) as the sole carbon source. The medium salts and the glycerol were autoclaved separately and then aseptically added to the fermentation vessel before inoculation. The inoculum was prepared by aseptically centrifuging the 1 L LB culture to separate the bacterial cells from the spent LB broth and the cells were then aseptically resuspended in medium *E** and added to the fermenter. Incubation was carried out for 4 days at 30°C with an impeller speed of 250 rpm and aeration at 2 L air/min and no pH control. At the appropriate time, cells were collected by centrifugation (8000 × *g*, 4°C, 15 min) and washed once with cold deionized water. Cells were then lyophilized to dryness and extracted in 600 mL of chloroform to separate the cellular material from the PHB polymer. This extraction was conducted at 30°C overnight with shaking at 250 rpm. The cellular material was removed by vacuum filtration through Whatman No. #2 filter paper and the chloroform fraction rotary-evaporated to concentrate the crude polymer. The crude polymer was then reprecipitated by dropwise addition into cold methanol. The pure polymer was recovered and dried *in vacuo* for 24 h.

Preparation of sophorolipid impregnated PHB- and PHB/HHx-based discs

The PHB-SL and PHB/HHx-SL discs were prepared by weighing out 5 separate 250 mg samples of either PHB or PHB/HHx polymer into separate 25 mL beakers (10 samples total). Sophorolipids were added to the beakers at varying concentrations. No SLs were added to the first set of polymer samples. These were the controls. To the second set of samples, 25 mg of SLs were added, to the third set of samples, 62.5 mg of SLs were added, to the fourth set of samples, 125 mg of SLs were added, and to the fifth set of samples, 187.5 mg of SLs were added. These combinations resulted in final SL concentrations of 0, 9.1, 20.0, 33.3, and 42.9 wt%, respectively, for both the PHB and PHB/HHx polymers. Ten milliliters of $CHCl_3$ was then added to each beaker containing the polymer-SL mixtures and the contents allowed to dissolve completely at which point the mixtures were poured into separate aluminum dishes (7.5 cm dia.) and the chloroform allowed to evaporate off at room temperature thus forming a thin film. The films were then placed under vacuum overnight to thoroughly remove any residual solvent and the discs were punched out from the polymer-SL films using either a cork borer or a paper hole-punch.

Preparation of sophorolipid impregnated pectin- and alginate-based discs

Films of pectin- and alginate-based SL composites were solution-cast. Briefly, 2.0 g pectin or alginate, 0.45 g hyaluronate and 0.8 g glycerol were mixed in water (75 mL for pectin- and 85 mL for alginate-based films). If needed, 3 mL of 5% SL in ethanol were dispersed with vigorous stirring in the resultant solutions. Hyaluronate was added in the formulation to improve the biocompatibility of the composite materials for biomedical applications as shown in a previous study [18]. Films were then cast at 1.9–2.2 mm wet thickness, and allowed to air dry. Crosslinking was achieved by soaking the films for two to three hours under gentle shaking in 75% ethanol containing 5% $Ca(OH)_2$ in Petri dishes. After decanting the solution, the films were washed 3 times with 50% ethanol and air-dried. Discs for antimicrobial testing were prepared from these films using a paper hole-punch.

Preparation of overlay plates and incubation conditions for *P. acnes*

Long-term stored *P. acnes* stock culture was thawed and used (0.2 mL) to inoculate 28 mL of a Difco™ Reinforced Clostridial Medium (herein D-RCM) in a sterile, screw-capped culture tube. The culture occupied almost the entire volume of the tube, providing a semi-anaerobic growth conditions for *P. acnes*. Cell growth was carried out by incubating the tube culture at 37°C without shaking for 2 days. Because *P. acnes* is a facultative anaerobe, the top portion of the tube culture was clear and devoid of cells. A 2 mL aliquot of the culture containing densely growing *P. acnes* was drawn from the bottom of the tube culture using a pipette and was added to 50 mL of freshly autoclaved and cooled (50°C) D-RCM-agar (1%, w/v) medium. The *P. acnes*-containing agar medium was poured into 10-cm Petri culture plates, allowed to solidify, and left inverted in a biological hood to dry overnight. Sophorolipid-laden biofilm discs (see previous section for their preparation) were overlaid on the *P. acnes*-containing solid agar-medium, and the culture plates were left overnight at room temperature to allow slow-release of SL while the *P. acnes* growth was minimal due to the ambient aerobic conditions. The plates were then transferred and sealed in Becton Dickinson GasPak™ EZ Anaerobe Gas Generating Pouches (BD Diagnostics, Franklin Lakes, NJ) and incubated at 37°C. After 2–4 days of incubation, the anti-*P. acnes* activity of SL was indicated by the appearance of a cell-free, clear halo around the biofilm disc.

Differential scanning calorimetry (DSC) of PHB and PHB/HHx

Thermal properties of PHB and PHB/HHx were measured using a Pyris 1 Differential Scanning Calorimeter (PerkinElmer, Norwalk, CT) at a heating rate of 10°C/min under a dry nitrogen purge. The instrument was calibrated using both indium (T_m = 156°C) and cyclohexane (transition temperatures at −87 and 6°C). Each sample was measured between −20 and 200°C and the T_m was taken as the peak of the melting endotherm.

Results and discussion

Sophorolipids have been documented to exhibit antimicrobial activities towards many different bacterial strains (primarily $Gram^+$) [11] and since they are simple glycolipids of natural origin, they have the added advantage of being biocompatible and biodegradable. *P. acnes* is a $Gram^+$ facultative anaerobe that is intimately associated with outbreaks of acne especially among adolescents. Therefore, it is conceivable that SLs can be used as effective anti-acne agents provided that they can be applied to the affected areas in a satisfactory manner. In this study we evaluated the effectiveness of using four different biopolymers (2 bacteria-derived polyesters, PHB and PHB/HHx and 2 plant-derived polysaccharides, pectin and alginate) as matrices for the application of SLs and deduced the concentration and structural dependence of SLs on the efficiency of action against *P. acnes*.

Poly(3-hydroxybutyrate) and PHB/HHx both proved to be successful as matrices to deliver SLs to *P. acnes*. Since SLs are relatively insoluble in water, organic solvents were used to produce the PHB- and PHB/HHx-SL composite films in preparation for testing. One advantage of using organic solvents was the ability to produce films with higher concentrations of SLs embedded in the polymer matrices. In fact, both the PHB- and PHB/HHx-SL composite films were prepared with varying concentrations of lactonic SLs up to 43% (w/w). In both instances, the composite films that contained no SL indicated no antimicrobial effects against *P. acnes* however; as the concentration of SLs increased in the polymer matrices, increased bacteriocidal action followed as shown by the increasing diameters of the zones of inhibition (Fig. 3). Interestingly, at 9.1% (w/w) SL in PHB no inhibitory action was observed (Fig. 3a) but that same concentration of SLs in PHB/HHx resulted in a small but noticeable clear zone developing around the edge of the disc (Fig. 3b). By measuring the distance from the edge of the discs to the edges of the zones of inhibition it was evident that the PHB/HHx polymer provided an improvement over PHB on the antimicrobial effects of SLs towards *P. acnes* (Fig. 4; it is worth noting also that the PHB/HHx polymer discs used in these experiments were smaller than the PHB polymer discs, and thus delivered less SL, but nonetheless resulted in slightly larger zones of inhibition). This improvement could be the result of an easier release of SLs due to an increased concentration of amorphous regions in the PHB/HHx polymer. It is well known that the incorporation of medium-chain monomers (C6 and higher) into a PHB polymer chain increases the amorphous character of the polymer. In fact, a comparison of the thermal properties (measured by DSC) of the PHB and PHB/HHx used in this study showed a decrease in melting temperature (T_m) from 173°C for PHB to 134°C for PHB/HHx and a corresponding 55% decrease in enthalpy of fusion (ΔH_f) from 19.4 cal/g for PHB to 8.8 cal/g for PHB/HHx (data not shown). These changes in thermal properties indicated an increased amorphous character in the PHB/HHx. The higher degree of chain randomness that generally defines the amorphous character of a polymer would tend to be less efficient in holding the SLs in the polymer matrix and therefore, would be expected to more easily liberate the SLs into the surrounding environment and improve the antimicrobial effect on *P. acnes*. One potential drawback to the use of PHB/HHx is the aesthetics of its use. As shown in Fig. 3, higher concentrations of SLs (20% and up) in the polymer matrices resulted in increased opacity. Since acne is generally located on the face and occurs at a time in life where self-esteem/image is important, opacity in the delivery system at least in areas that are visible are less than ideal.

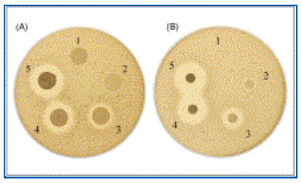


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Figure 3.

Images of overlay plates containing *P. acnes* and SL composite film discs composed of PHB **(a)** and PHB/HHx **(b)**. Sophorolipid (SL) concentrations (w/w) in the discs were as follows: 0 SL (1), 9.1% SL (2), 20.0% SL (3), 33.3% SL (4), and 42.9% SL (5).

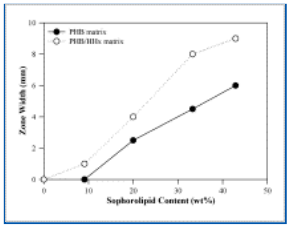

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Figure 4.

Graph of the comparative anti-*P. acnes* effects of SLs as a function of SL concentration in the polymer film matrix and the type of poly(hydroxyalkanoate) polymer used in the composite.

The apparent drawback (described previously) to using either PHB or PHB/HHx as polymer delivery systems for SLs against *P. acnes* caused a shift in the focus of our research towards more transparent polymers including the plant-derived polysaccharides, pectin and alginate. Fig. 5 (sectors 1 and 2) shows that both pectin and alginate films, in the absence of SLs, did not exhibit an inhibitory effect on the growth of *P. acnes* cells. In fact, a slight stimulatory effect on cell growth could be argued for the A-H-G (for abbreviation definition see Fig. 5 legend) composite film, as the edge of the discs in sector 2 of Fig. 5 can be seen to have a higher concentration of cells in comparison to their surrounding. We do not have an explanation for the possible stimulatory effect of A-H-G film on bacterial growth. The inhibition of *P. acnes* cell growth by SL embedded in the two composite films is shown in sectors 3 and 4 of Fig. 5. On the basis of the comparative diameters of the zones of inhibition between the pectin- and alginate-based composite films, we concluded that the two film types did not differ in their capability to release the embedded SL. The physical appearance of the two composite-film types allows for different applications for these antimicrobial materials. Unlike the PHB and PHB/HHx films, the A-H-G films remained transparent even after SL addition. Thus, it is envisioned that A-H-G films may be beneficial for applications where appearance is an important consideration such as in facial anti-acne treatment in which it is preferred to have a less visible medium. The opaque P-H-G film, on the other hand, provides a cost-effective alternative SL carrier for antimicrobial applications in which light-shielding may be preferable, such as in wrapping films.

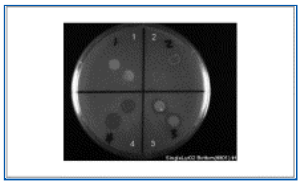

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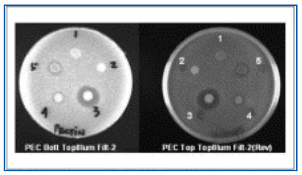
Figure 5.

Image of overlay plates containing *P. acnes* and SL composite film discs composed of pectin and alginate films. Compositions of the biocomposite films are as follows: pectin (P, 61.4%)-hyaluronate (H, 13.8%)-glycerol (G, 24.8%; *Zone 1*), alginate (A, 61.4%)-H-G (*Zone 2*), P-H-G containing SL (0.24%; *Zone 3*), A-H-G containing SL (0.24%; *Zone 4*). The sophorolipid sample is mainly the lactonic species (>80%).

We next compared the anti-*P. acnes* activities of the lactonic and open-chain free-acid forms of SLs. Furthermore, to improve the handling properties of the composite films, we crosslinked the films by

subjecting them to an ethanol/$Ca(OH)_2$ treatment (see 'materials and methods' section). Fig. 6 summarizes the results of the study with pectin-based composite films. Three conclusions can be drawn from these data. First, the crosslinking treatment increased the opacity of the pectin-based composite films, as evidenced by a comparison of the images of discs 1 (non-crosslinked) and 2 (crosslinked), and of discs 4 (crosslinked) and 5 (non-crosslinked) in Fig. 6. Second, lactonic SL exhibited a stronger anti-*P. acnes* activity (disc 3) when compared to the free-acid form of SL (discs 4 and 5). This is evident from the large diameter of the zone of inhibition around disc 3 (Fig. 6). Finally, the crosslinking treatment caused a decline in the release of the open-chain, free-acid SL, as a comparison of the appearance of the clear halos around discs 4 (crosslinked) and 5 (non-crosslinked) revealed. It seems that crosslinking had toughened the films to reduce breakage on handling but also had resulted in a tighter fibril network that hampered the release of SL into the medium.

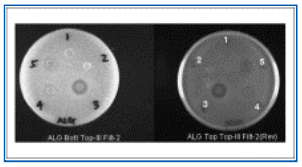


Full-size image (33K)

Figure 6.

Images of overlay plates containing *P. acnes* and SL composite film discs showing the comparative antimicrobial activity of open-chain vs. lactonic sophorolipids in pectin-based biocomposite films. See legend for Fig. 5 for abbreviations and compositions. P-H-G (*disc 1*), crosslinked (XL-) P-H-G (*disc 2*), XL-P-H-G containing predominantly lactonic SL (*disc 3*), XL-P-H-G containing open-chain free-acid form of SL (*disc 4*), P-H-G containing open-chain free-acid form SL (*disc 5*). *Left panel:* viewed from the bottom of the culture plate, *right panel:* viewed from the top of the culture plate.

A similar comparative study of the anti-*P. acnes* activities of the two forms (i.e., lactonic and open-chain free-acid) of SL embedded in the alginate-based films (crosslinked and non-crosslinked) was conducted. Fig. 7 presents the results of the investigation, which essentially followed those observed with the pectin-based films. The transparency of the alginate-based films is again apparent, even with disc 3 which is a crosslinked material. However, when compared to the observation in Fig. 5, crosslinking had in fact introduced a slight opacity to the alginate film.



Full-size image (29K)

Figure 7.

Images of overlay plates containing *P. acnes* and SL composite film discs showing the comparative antimicrobial activity of open-chain vs. lactonic sophorolipids in alginate-based biocomposite films. See legend for Fig. 5 for abbreviations and compositions. A-H-G (*disc 1*), XL-A-H-G (*disc 2*), XL-A-H-G containing mostly lactonic SL (*disc 3*), XL-A-H-G containing open-chain free-acid form of SL (*disc 4*), A-H-G containing open-chain free-acid form SL (*disc 5*). *Left panel:* viewed from the bottom of the culture plate, *right panel:* viewed from the top of the culture plate.

When we compared the dose-response relationships between the SL contents of the four types of films and their anti-*P. acnes* activity, we noted that a lower concentration of SL was needed in pectin- and alginate-based composites to elicit the appearance of a clearing zone. In PHB-based films, SL content as high as 9% did not exhibit antimicrobial activity in our assay system. In the plant-based composites, however, the cell-free clear halo was observed at a SL content as low as 0.24%. This interesting observation indicated that perhaps the pectin- and alginate-based materials are better suited for applications that require moderate to fast release of SL, while those based on PHA can be used for slow-release applications.

In conclusion, biopolymer-embedded SLs were demonstrated to exert antimicrobial effects on the acne-causing bacterium, *P. acnes* with varying success rates based on the specific biopolymer matrix. While PHB/HHx showed the most success based on the diameter of the zone of inhibition, the opacity of the PHB/HHx-SL films at high SL concentrations made them less attractive as potentially visible topical treatments for acne. By contrast, the pectin- and alginate-based SL composite films, whether crosslinked or not, were also effective against *P. acnes*. Although the SL

concentrations in these films were substantially lower, antimicrobial activity, especially among the lactonic form of the SLs, was present as witnessed by the occurrence of noticeable zones of inhibition. An added benefit to the use of pectin- and alginate-based films was the maintenance of a more acceptable level of polymer transparency making them potentially more acceptable for visible, topical applications. The use of SLs as anti-acne agents presents several advantages over currently used remedies such as limited potential skin irritation and bleaching as currently experienced with benzoyl peroxide, no development of bacterial resistance as typically occurs with antibiotic treatments, and SLs may be less expensive than hormonal treatments. In addition, SLs are renewable, and environmentally benign therefore; by using biopolymer matrices that are also environmentally friendly, as delivery carriers a new potentially large niche market can be tapped thus benefiting the consumer, the environment, the SL producer and the polymer producer/processor.

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