

***IN VITRO* CHRONIC WOUND MODEL**

TECHNICAL FIELD

The present disclosure relates to a novel *in-vitro* bacteria-infected chronic wound model for robust screening of anti-microbials and wound healing agents. Also provided are a method of producing the wound model, a screening platform comprising one or more wound models, and a method of assessing the activity of an agent using the wound model.

BACKGROUND

Infectious diseases, like chronic wounds, possess a significant threat to the public health systems due to increase in antimicrobial resistance (AMR), inefficacy of antibiotics, dearth of novel biologics and off-target effects leading to the normal flora damage. Chronic wounds are a large and growing problem predominantly due to the emergence of multidrug resistant (MDR) bacterial pathogens with a reported annual spending of US\$25 billion in United States¹⁻². Another big challenge is the lack of optimal, reproducible bacteria-infected *in-vitro/ex-vivo* models for effective preclinical screenings of antimicrobials and wound-healing candidates. This lack of knowledge has arisen in part from the less than ideal *in vitro* and *ex-vivo* models available for infection-related skin research³. Animal models which are heavily used for preclinical testing have come under increasing scrutiny for ethical and scientific reasons such as non-human like mode of healing (murine models), expenses and logistics (porcine models)³. Furthermore, most of these models do not represent infected chronic wound environment. To date, there is a lack of an *in-vitro* wound model in which the efficacy of potential candidates can be co-investigated both in terms of wound healing and bacterial clearance at the wound site.

Thus, it is critical to develop a robust screening platform for candidates that not only address wound healing potential but also recapitulate chronic wound environment wherein crosstalk between bacteria and skin cells occurs, simulating an infection state. In doing so, such platform will enable reliable testing of novel therapeutics, providing insights toward treatment strategies against chronic wounds.

SUMMARY

In one aspect, there is provided an *in vitro* chronic wound model, comprising a wound region surrounded by skin cells, wherein the wound region comprises a bacteria
5 cellulose (BC) pellicle, which has a biofilm comprising one or more species of bacteria.

In one embodiment, the model further comprises a cell culture vessel which contains the pellicle and the skin cells, for example wherein the skin cells are grown on the surface of the cell culture vessel.

In one embodiment, the model further comprises a device which forms a barrier
10 between the pellicle and the skin cells, thereby preventing the skin cells from growing into the wound region.

In one aspect, there is provided a method of producing an *in vitro* chronic wound model comprising a wound region surrounded by skin cells, comprising the steps of:

- a) inoculating a bacterial cellulose (BC) pellicle with one or more species of
15 bacteria so as to form a biofilm;
- b) growing a plurality of skin cells in a cell culture vessel; and
- c) introducing the pellicle into the cell culture vessel, thereby forming the wound region.

In one embodiment, the pellicle is sterilized, for example by autoclaving, prior to
20 inoculation with the one or more species of bacteria.

In one embodiment, the pellicle is inoculated with bacteria for at least 48 hours, for example 48, 60 or 72 hours, in particular for 47 hours.

In one embodiment, the pellicle is washed, for example using a biologically compatible buffer such as PBS, to remove any non-adherent bacteria prior to step c).

25 In one embodiment, the method further comprises the step of introducing a device which forms a barrier between the pellicle and the skin cells prior to step b), thereby preventing the skin cells from growing into the wound region.

In one embodiment, the skin cells are grown for 2 or more days, such as 2, 3 or 4 days, in particular for 3 days.

30 In one embodiment, the method further comprises the step of introducing an agent to prevent the skin cells from growing into the wound region, for example wherein the agent is trypsin or a derivative thereof, such as TrypLE™ Express.

In one embodiment, the method further comprises the step of removing the device after step c).

In one embodiment, the skin cells are derived from a mammalian organism, for example a primate, a domesticated animal or a livestock animal, for example selected from the group comprising a human, a monkey, a dog, a cat, a sheep, a goat, a cow, or a horse, in particular human skin cells.

5 In one embodiment, the skin cells are keratinocytes.

In one embodiment, the biofilm comprises only one species of bacteria.

In one embodiment, the biofilm comprises multiple species of bacteria, i.e. at least two different species of bacteria.

In one embodiment, the one or more species of bacteria are pathogenic bacteria, for example wherein the bacteria are from a bacterial genus selected from the group comprising: *Bacillus*, *Bartonella*, *Bordetella*, *Borrelia*, *Brucella*, *Campylobacter*, *Chlamydia*, *Chlamydophila*, *Clostridium*, *Corynebacterium*, *Enterococcus*, *Escherichia*, *Francisella*, *Haemophilus*, *Helicobacter*, *Legionella*, *Leptospira*, *Listeria*, *Mycobacterium*, *Mycoplasma*, *Neisseria*, *Pseudomonas*, *Rickettsia*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, *Treponema*, *Ureaplasma*, *Vibrio* and *Yersinia*.

In one embodiment, the one or more species of bacteria are selected from the group comprising: *Acinetobacter baumannii*, *Bacillus anthracis*, *Bacillus cerues*, *Bartonella henselae*, *Bartonella quintana*, *Bordetella pertussis*, *Borrelia burgdorferi*, *Borrelia garinii*, *Borrelia afzelii*, *Borrelia recurrentis*, *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella suis*, *Campylobacter jejuni*, *Chlamydia pneumoniae*, *Chlamydia trachomatis*, *Chlamydophila psittaci*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium tetani*, *Corynebacterium diphtheriae*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterobacter spp*, *Escherichia coli*, *Francisella tularensis*, *Hemophilus influenzae*, *Helicobacter pylori*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Leptospira interrogans*, *Leptospira santarosai*, *Leptospira weilli*, *Leptospira noguchii*, *listeria monocytogenes*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Mycobacterium ulcerans*, *Mycoplasma pneumoniae*, *Neisseira gonorrhoeae*, *Neisseira meningitidis*, *Pseudomonas aeruginosa*, *Rickettsia rickettsia*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Treponema pallidum*, *Ureaplasma urealyticum*, *Vibrio cholerae*, *Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*, in particular wherein the one or more species of bacteria are selected from the group comprising *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Komagataeibacter hansenii*.

In one embodiment, the cell culture vessel is a tissue culture plate, for example a 6, 12, 24 or 96 well tissue culture plate, in particular a 24-well tissue culture plate.

In one embodiment, the device comprises a fence and a collar, for example as shown in Figure 2A.

5 In one embodiment, the device is located in a well of the tissue culture plate, thereby creating an inner and an outer well.

In one embodiment, the pellicle is in the inner well and the skin cells are in the outer well.

10 In one embodiment, the model further comprises one or more additional cell types, such as senescent cells for example induced by introducing Etoposide to the cells.

In one embodiment, the model has one or more of the following environments: an alkaline pH environment, for example achieved by introducing CO₂, such as 1% CO₂ into the model; a hypoxic environment, for example achieved by introducing cobalt chloride, such as 20 µM CoCl₂ into the model; and a high oxidative stress environment,
15 for example achieved by introducing Etoposide into the model.

In one aspect, there is provided a screening platform, such as a high throughput screening platform, comprising one or more *in vitro* chronic wound models as defined above.

20 In one aspect, there is provided a method of assessing the activity of an agent, comprising the steps of:

- exposing an *in vitro* chronic wound model according to any preceding claim to the agent, followed by either one or both of the following steps:
- determining one of more of the following:
 - the extent of migration,
 - 25 • the rate of migration, and
 - the delay in migration

of the skin cells into the wound region of the model; and/or

- performing a count of the viable bacteria present in the pellicle.

30 In one embodiment, the method further comprises step d) comparing the migration measurements and/or colony counts with the migration measurements and/or colony counts of an *in vitro* chronic wound model according to any preceding claim that has been exposed to a control agent.

35 In one embodiment, the activity is selected from the group comprising anti-microbial activity, wound healing activity, and both anti-microbial and wound healing activity.

In one embodiment, the agent is selected from the group comprising an anti-microbial agent, a wound healing agent, and an agent with anti-microbial and wound healing properties.

In one embodiment, the extent, rate and/or delay of migration is determined by
5 imaging the model, for example by using time lapse microscopy

In one embodiment, the extent, rate and/or delay of migration is determined by monitoring the leading edge of the wound over time, for example by determining the % wound closure.

In one embodiment, the pellicle is sonicated prior to the viable count.

10 In one embodiment, the control agent is selected from the group comprising media, a wound healing control and an antibacterial agent.

In one embodiment, the wound healing control is selected from the group comprising: a growth factor, for example epidermal growth factor (EGF) or vascular endothelial growth factor (VEGF), fibronectin and vitronectin.

15 In one embodiment, the antibacterial agent is selected from the group comprising: an antibiotic, such as gentamicin; an antimicrobial peptide, for example selected from the group comprising ENO3, SPARCL1 and V8LQ19L; an antimicrobial polymer; and an extracellular vesicle (EV), such as an adipose stem cell EV (ASC-EV).

20 DEFINITIONS

The term "chronic wound" as used herein refers to wound that does not proceed through an orderly and timely reparative process. A wound that does not heal within 3 months is typically considered chronic. Chronic wounds generally have one or more of the following characteristics:

- 25
- Fail to Progress: They do not proceed through an orderly or timely reparative process.
 - Prolonged Inflammation: The majority of chronic wounds exhibit a prolonged or excessive inflammatory phase.
 - Cell Responsiveness Impairment: Dermal or epidermal cells struggle to
30 respond to reparative stimuli.
 - Persistent Infections: Microbial squatters further complicate matters.

The majority of chronic wounds can be classified under three categories: venous, diabetic and pressure ulcers. Other less common categories include wounds due to radiation poisoning and ischemia.

35 Treatment strategies for chronic wounds are varied and include:

- Local care: For example, debridement to aggressively remove necrotic debris or non-viable tissue, and proper wound dressing.
- Addressing the underlying causes: For example, treating comorbidities such as diabetes; weight management since excess weight can impair wound healing; and reviewing the patient's existing treatments, for example long term use of immunosuppressants may hinder wound healing
- Pain management: chronic wounds are often associated with chronic pain
- Infection control: For example, by using anti-microbial agents.
- Wound closure: For example, employing skin grafts to help cover the wound

10 The term "wound region" as used within the context of the disclosure refers to the part/section/region of the *in vitro* model which mimics a wound.

The term "bacterial cellulose" or BC as used herein refers to an organic compound having the formula $(C_6H_{10}O_5)_n$, which is synthesized by specific bacterial genera, such as *Komagataeibacter*, *Acetobacter*, *Sarcina ventriculi* and *Agrobacterium*.

15 BC differs from plant cellulose in that it is more chemically pure, containing no hemicellulose or lignin, has higher tensile strength and has an increased water-holding capacity. In addition, because BC has a more crystalline structure than plant cellulose, it has higher moldability and can be formed into virtually any shape.

Due to its unique properties, BC has numerous different applications. For example, it is added to many food products as a dietary fiber, is used as an additive in cosmetics, and is even used in acoustic/filter membranes in loudspeakers and headphones due to its sonic velocity and low dynamic loss. Within the field of medicine, BC is used wound dressings, drug delivery carriers and tissue engineering scaffolds.

25 The term "pellicle" as used herein refers to a thin skin, film or membrane. Thus, the term "bacterial cellulose pellicle" is intended to refer to a thin skin, film or membrane composed of bacterial cellulose.

The term "biofilm" as used herein refers to a syntrophic assembly of microorganisms, such as bacteria, fungi and/or algae, that stick to each other and often adhere to surfaces. The adherent cells create a 3D structure, embedded within a slimy extracellular matrix composed of extracellular polymeric substances (EPSs).

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Biofilms have been found to be involved in a wide range of microbial infections in the body and it has been suggested that around two-thirds of all bacterial infections in humans involve the formation of biofilms. Infectious processes in which biofilms have been implicated include common minor infections, such as urinary tract infections, middle-ear infections, the formation of dental plaque; as well as more serious and rarer

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infections. Such as endocarditis, infections in cystic fibrosis and infections of medical implants such as heart valves and joint prostheses.

Infections involving biofilms are particularly challenging to eradicate, due to the fact that mature biofilms display various traits such as antimicrobial tolerance and immune response evasions. In the context of chronic wounds, the presence of biofilms hinder wound healing, delay tissue repair and stimulate chronic inflammation at the wound site.

The term "skin cells" as used herein refers to any type of cell involved in the formation of the epidermis and dermis, which are the outer two layers which make up the skin. Examples of skin cells include but are not limited to:

- Keratinocytes, which are the most abundant skin cell type. Their primary role is to create a tough waterproof layer to protect the body from harmful chemicals, UV radiation and infectious agents
- Melanocytes, which are responsible for skin pigmentation. Their primary function is to produce melanin which absorbs and dissipates UV radiation
- Langerhans cells, which are immune cells found in the epidermis. They play an important role in detecting and responding to pathogens
- Merkel cells, which are sensory cells which allow the body to perceive touch.

The term "cell culture vessel" as used herein refers to a container designed for the growth and maintenance of cells outside their natural environment. Cell culture vessels generally provide the following:

- Suitable growth environment: cell culture vessels provide an artificial environment which mimics the natural conditions required for cell proliferation and survival. This may for example include the regulation of pH, osmotic pressure, temperature, etc.
- Sterile environment: cell culture vessels provide a sterile environment to minimise the risk of contamination
- Essential nutrients: cell culture vessels generally contain a growth media which contains the nutrients required for cell survival and growth.
- Gaseous exchange: cell culture vessels typically allow for the exchange of gases (e.g. oxygen and carbon dioxide) vital for cell metabolism.

Examples of types of cell culture vessels include but are not limited to petri dishes, tissue culture flasks, spinner flasks and multi-well plates.

The term "fence" as used herein refers to a structure that functions as a barrier within a well of a cell culture vessel to prevent cells or microorganisms from growing or

migrating past it. When placed within a well, it subdivides the well into an inner well and an outer well. Cells/microorganisms placed in the inner well are unable to leak or migrate to the outer well and vice versa.

The term "collar" as used herein is a structure that is designed and fitted to the "fence". Its main function is to enable the fence to be returned to its original position within a cell culture well after the fence has been previously removed. In particular, the collar increases the fence circumference to precisely fit the well, thereby helping to eliminate lateral displacement of the fence in the outer-well during removal and replacement.

A more detailed discussion of the "fence and collar" design can be found in Wager et al, Cell Ah Migr 2017; 11(5-6): 496-503.

The term "and/or", e.g., "X and/or Y" is understood to mean either "X and Y" or "X or Y" and should be taken to provide explicit support for both meanings or for either meaning.

Further, in the description herein, the word "substantially" whenever used is understood to include, but not restricted to, "entirely" or "completely" and the like. In addition, terms such as "comprising", "comprise", and the like whenever used, are intended to be non-restricting descriptive language in that they broadly include elements/components recited after such terms, in addition to other components not explicitly recited. For example, when "comprising" is used, reference to a "one" feature is also intended to be a reference to "at least one" of that feature. Terms such as "consisting", "consist", and the like, may in the appropriate context, be considered as a subset of terms such as "comprising", "comprise", and the like. Therefore, in embodiments disclosed herein using the terms such as "comprising", "comprise", and the like, it will be appreciated that these embodiments provide teaching for corresponding embodiments using terms such as "consisting", "consist", and the like. Further, terms such as "about", "approximately" and the like whenever used, typically means a reasonable variation, for example a variation of +/- 5% of the disclosed value, or a variance of 4% of the disclosed value, or a variance of 3% of the disclosed value, a variance of 2% of the disclosed value or a variance of 1% of the disclosed value.

Furthermore, in the description herein, certain values may be disclosed in a range. The values showing the end points of a range are intended to illustrate a preferred range. Whenever a range has been described, it is intended that the range covers and teaches all possible sub-ranges as well as individual numerical values within that range. That is, the end points of a range should not be interpreted as inflexible limitations. For

example, a description of a range of 1% to 5% is intended to have specifically disclosed sub-ranges 1% to 2%, 1% to 3%, 1% to 4%, 2% to 3% etc., as well as individually, values within that range such as 1%, 2%, 3%, 4% and 5%. It is to be appreciated that the individual numerical values within the range also include integers, fractions and
5 decimals. Furthermore, whenever a range has been described, it is also intended that the range covers and teaches values of up to 2 additional decimal places or significant figures (where appropriate) from the shown numerical end points. For example, a description of a range of 1% to 5% is intended to have specifically disclosed the ranges
10 1.00% to 5.00% and also 1.0% to 5.0% and all their intermediate values (such as 1.01%, 1.02% ... 4.98%, 4.99%, 5.00% and 1.1%, 1.2% ... 4.8%, 4.9%, 5.0% etc.) spanning the ranges. The intention of the above specific disclosure is applicable to any depth/breadth of a range.

Additionally, when describing some embodiments, the disclosure may have disclosed a method and/or process as a particular sequence of steps. However, unless
15 otherwise required, it will be appreciated that the method or process should not be limited to the particular sequence of steps disclosed. Other sequences of steps may be possible. The particular order of the steps disclosed herein should not be construed as undue limitations. Unless otherwise required, a method and/or process disclosed herein should not be limited to the steps being carried out in the order written. The sequence of steps
20 may be varied and still remain within the scope of the disclosure.

Furthermore, it will be appreciated that while the present disclosure provides embodiments having one or more of the features/characteristics discussed herein, one or more of these features/characteristics may also be disclaimed in other alternative embodiments and the present disclosure provides support for such disclaimers and
25 these associated alternative embodiments.

DESCRIPTION OF EMBODIMENTS

It will be appreciated by a person skilled in the art that other variations and/or modifications may be made to the embodiments disclosed herein without departing
30 from the spirit or scope of the disclosure as broadly described. For example, in the description herein, features of different exemplary embodiments may be mixed, combined, interchanged, incorporated, adopted, modified, included etc. or the like across different exemplary embodiments. The present embodiments are, therefore, to be considered in all respects to be illustrative and not restrictive.

Existing *in-vitro* and *ex-vivo* models do not represent chronic wound settings, as they do not contain bacterial biofilms at the wound site. The biggest hurdle in the implementation of a bacteria-infected wound model is to contain the bacteria at the wound site.

5 The present inventors have overcome this challenge by placing bacteria cellulose (BC) pellicle containing pre-formed biofilms into the centred wound region surrounded by keratinocytes to mimic bacterial burden at the wound site. Thus, the present inventors have successfully established an *in-vitro* bacteria-infected chronic wound model wherein, sustained coexistence and interdependency of bacterial biofilms at the wound
10 site and surrounding keratinocytes mimics a chronic wound setting.

In addition, the disclosed *in-vitro* model that circumvents the need to have human scaffolds. This reduces the overall cost of the technology. The technology uses an abiotic surface for biofilm growth in a closed environment from keratinocytes thus circumventing competitive growth between keratinocytes and microbial growth. This feature enables
15 monitoring of wound closure in the presence of mature biofilms thus facilitating candidate screening and validation.

Thus, in one aspect, there is provided an *in vitro* chronic wound model, comprising a wound region surrounded by skin cells, wherein the wound region comprises a bacteria cellulose (BC) pellicle, which has a biofilm comprising one or more
20 species of bacteria.

In one embodiment, the model further comprises a cell culture vessel which contains the pellicle and the skin cells. The advantage of the inclusion of the cell culture vessel is that it provides a suitable and sterile environment for culturing the skin cells, thereby optimising skin cell growth and minimising the risk of the model becoming
25 contaminated.

In one embodiment, the skin cells are grown on the surface of the cell culture vessel.

In one embodiment, the model further comprises a device which forms a barrier between the pellicle and the skin cells, thereby preventing the skin cells from growing
30 into the wound region. Advantageously, the device helps to create a more clearly defined wound edge, which separates the wound region from the surrounding healthy skin cells.

In one aspect, there is provided a method of producing an *in vitro* chronic wound model comprising a wound region surrounded by skin cells, comprising the steps of:

- 35 a) inoculating a bacterial cellulose (BC) pellicle with one or more species of bacteria so as to form a biofilm;
b) growing a plurality of skin cells in a cell culture vessel; and

c) introducing the pellicle into the cell culture vessel, thereby forming the wound region.

Advantageously, the presently disclosed method provides a simple and efficient way of producing an *in vitro* chronic wound model that accurately mimics a chronic wound.

In one embodiment, the pellicle is sterilized, for example by autoclaving, prior to inoculation with the one or more species of bacteria. Advantageously, sterilising the pellicle helps to minimise the risk of the pellicle being contaminated with other pathogens besides the target bacteria species.

In one embodiment, the pellicle is inoculated with bacteria for at least 48 hours, for example 48, 60 or 72 hours. The present inventors have established that bacteria grown for less than 48 hours, such as 24 hours, exhibit reduced biofilm mass. Thus, prolonging the infection duration to 48 hours or more helps to achieve thicker/denser biofilms.

In one embodiment, the pellicle is inoculated with bacteria for 48 hours.

In one embodiment, the pellicle is washed, for example using a biologically compatible buffer, to remove any non-adherent bacteria prior to step c).

In one embodiment, the pellicle is washed with PBS prior to step c).

In one embodiment, the method further comprises the step of introducing a device which forms a barrier between the pellicle and the skin cells prior to step b), thereby preventing the skin cells from growing into the wound region. Advantageously, introducing the device creates a physical barrier that helps to create a more clearly defined wound edge, which separates the wound region from the surrounding healthy skin cells.

In one embodiment, the skin cells are grown for 2 or more days, such as 2, 3 or 4 days. As described above, inoculating the bacteria for 48 hours or more helps to achieve a thicker biofilm. Thus, by growing the skin cells for 2 or more days (i.e. also 48 hours or more), this helps to synchronise the growth of skin cells with the production of the biofilm in the wound region, thereby eliminating delays and streamlining the production of the chronic wound model.

In one embodiment, the skin cells are grown for 3 days.

In one embodiment, the method further comprises the step of introducing an agent to prevent the skin cells from growing into the wound region. Advantageously, the agent acts in concert with the device to prevent skin cells from growing into the wound region, wherein the device physically prevents the cells from growing into the wound region, while the agent chemically prevents the cells from growing into the wound region.

In one embodiment, the agent for preventing the skin cells from growing into the wound region is trypsin or a derivative thereof such as TrypLE™ Express.

In one embodiment, the method further comprises the step of removing the device after step c). This step removes the physical barrier normally preventing the skin
5 cells from growing into the wound region, thus permitting wound closure to occur.

In one embodiment, the skin cells are derived from a mammalian organism, for example a primate, a domesticated animal or a livestock animal.

In one embodiment, the skin cells are derived from a mammalian organism selected from the group comprising a human, a monkey, a dog, a cat, a sheep, a goat,
10 a cow, or a horse.

In one embodiment, the skin cells are human skin cells.

In one embodiment, the skin cells are keratinocytes.

In one embodiment, the biofilm comprises only one species of bacteria.

In one embodiment, the biofilm comprises multiple species of bacteria, i.e. at least
15 two different species of bacteria. Advantageously, this enables the production of a poly-microbial biofilm that allows the model to mimic chronic wounds infected by multiple bacteria species, for example chronic ulcers, such as chronic venous leg ulcers, which are frequently colonised by more than one bacteria species. In addition, this allows the model to be used for high throughput screening to assess the activity of antimicrobials
20 and/or wound healing agents against multiple bacteria species.

In one embodiment, the one or more species of bacteria are pathogenic bacteria.

In one embodiment, the one or more species of bacteria are from a bacterial genus selected from the group comprising: *Bacillus*, *Bartonella*, *Bordetella*, *Borrelia*, *Brucella*, *Campylobacter*, *Chlamydia*, *Chlamydophila*, *Clostridium*, *Corynebacterium*,
25 *Enterococcus*, *Escherichia*, *Francisella*, *Haemophilus*, *Helicobacter*, *Legionella*, *Leptospira*, *Listeria*, *Mycobacterium*, *Mycoplasma*, *Neisseria*, *Pseudomonas*, *Rickettsia*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, *Treponema*, *Ureaplasma*, *Vibrio* and *Yersinia*.

In one embodiment, the one or more species of bacteria are selected from the
30 group comprising: *Acinetobacter baumannii*, *Bacillus anthracis*, *Bacillus cerues*, *Bartonella henselae*, *Bartonella quintana*, *Bordetella pertussis*, *Borrelia burgdorferi*, *Borrelia garinii*, *Borrelia afzelii*, *Borrelia recurrentis*, *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella suis*, *Campylobacter jejuni*, *Chlamydia pneumoniae*, *Chlamydia trachomatis*, *Chlamydophila psittaci*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium tetani*, *Corynebacterium diphtheriae*,
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Enterococcus faecalis, *Enterococcus faecium*, *Enterobacter* spp, *Escherichia coli*, *Francisella tularensis*, *Hemophilus influenzae*, *Helicobacter pylori*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Leptospira interrogans*, *Leptospira santarosai*, *Leptospira weilli*, *Leptospira noguchii*, *listeria monocytogenes*, *Mycobacterium leprae*,
 5 *Mycobacterium tuberculosis*, *Mycobacterium ulcerans*, *Mycoplasma pneumoniae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Rickettsia rickettsia*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Treponema pallidum*,
 10 *Ureaplasma urealyticum*, *Vibrio cholerae*, *Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*.

In one embodiment, the one or more species of bacteria are selected from the group comprising *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*. Advantageously, these species of bacteria are amongst the most commonly
 15 found ones in chronic wounds. Thus, by including 1, 2 or all 3 species, the model can successfully mimic a large proportion of chronic wounds typically found in patients.

In one embodiment, the one or more species of bacteria are selected from the group comprising *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Komagataeibacter hansenii*.

20 In one embodiment, the one or more species of bacteria are selected from the group comprising *Enterococcus faecalis* and *Pseudomonas aeruginosa*. Advantageously, the present inventors have successfully demonstrated that production and use of *in vitro* chronic wound models comprising these bacteria species.

In one embodiment, the cell culture vessel is a tissue culture plate, for example
 25 a 6, 12, 24 or 96 well tissue culture plate. The advantage of employing a tissue culture plate is that this particular type of cell culture vessel generally comprises multiple defined wells, each of which can contain a chronic wound model of the present disclosure. This facilitates a wide range of different screening setups: for example, the screening of multiple anti-microbial and/or wound healing agents using a single plate, and/or the
 30 potential to include wound models having different environments on a single plate.

In one embodiment, the cell culture vessel is a 24-well tissue culture plate.

In one embodiment, the device comprises a fence and a collar, for example as shown in Figure 2A. Advantageously, the fence and collar is an established device design wherein the fence effectively subdivides a tissue culture well into an inner and an

outer well, and the collar facilitates the removal and re-insertion of the fence, thus enabling the removal and re-establishment of the inner well as required.

In one embodiment, the device is located in a well of the tissue culture plate, thereby creating an inner and an outer well.

5 In one embodiment, the pellicle is in the inner well and the skin cells are in the outer well. Advantageously, the placement of the pellicle relative to the skin cells helps to create a wound region surrounded by skin cells.

In one embodiment, the model or method further comprises one or more additional cell types. This has the benefit of enabling additional cell types to be included
10 in the wound model, thereby increasing the utility and/or enabling the wound model to more accurately mimic the complex physiology and dynamics of actual skin.

In one embodiment, the additional cells are senescent cells. Advantageously, chronic wounds typically include senescent cells. Thus, the inclusion of this extra cell type allows the model to mimic actual chronic wounds more accurately.

15 In one embodiment, the senescent cells are induced by introducing Etoposide to the cells.

In one embodiment, the model has one or more of the following environments: an alkaline pH environment, a hypoxic environment, and a high oxidative stress environment. Advantageously, chronic wounds often exhibit one or more of these
20 features. Hence, by modelling one or more of these environments in addition to bacterial burden, the usefulness and accuracy of the chronic wound model can be enhanced.

In one embodiment, the alkaline pH environment is achieved by introducing CO₂, such as 1% CO₂ into the model.

In one embodiment, the hypoxic environment is achieved by introducing cobalt chloride, such as 20 µM CoCl₂ into the model.
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In one embodiment, the high oxidative stress environment is achieved by introducing Etoposide into the model.

In one aspect, there is provided a screening platform, such as a high throughput screening platform, comprising one or more *in vitro* chronic wound models as described
30 above. Advantageously, the screening platform may comprise multiple *in vitro* chronic wound models, thereby enabling high throughput screening of multiple antibacterial and/or wound healing agents.

In one aspect, there is provided a method of assessing the activity of an agent, comprising the steps of:

a) exposing an *in vitro* chronic wound model as described herein to the agent, followed by either one or both of the following steps:

b) determining one or more of the following:

- the extent of migration,
- 5 • the rate of migration, and
- the delay in migration

of the skin cells into the wound region of the model; and/or

c) performing a count of the viable bacteria present in the pellicle.

Advantageously, the presently disclosed method provides an efficient way to
10 assess the activity of a given agent by employing the *in vitro* chronic wound model of the present disclosure.

In one embodiment, the method further comprises step d) comparing the migration measurements and/or colony counts with the migration measurements and/or colony counts of an *in vitro* chronic wound model as described that has been exposed to
15 a control agent. This step has the benefit of allowing the activity of the agent to be compared with the activity of an agent which has a known level of activity, thereby allowing agents with desired antimicrobial and/or wound healing activity to be identified and compared with known antimicrobial and/or wound healing agents.

In one embodiment, the activity is selected from the group comprising anti-
20 microbial activity, wound healing activity, and both anti-microbial and wound healing activity.

In one embodiment, the agent is selected from the group comprising an anti-microbial agent, a wound healing agent, and an agent with anti-microbial and wound healing properties.

25 In one embodiment, the extent, rate and/or delay of migration is determined by imaging the model, for example by using time lapse microscopy. Advantageously, imaging the model allows the extent, rate and/or delay of migration to be accurately determined and quantified.

In one embodiment, the extent, rate and/or delay of migration is determined by
30 monitoring the leading edge of the wound over time.

In one embodiment, the activity is assessed by determining the %wound closure.

In one embodiment, the pellicle is sonicated prior to the viable count. Advantageously, sonicating the pellet efficiently releases the biofilm from the pellicle, thereby allowing viable cell counts to be obtained.

In one embodiment, the control agent is selected from the group comprising media, a wound healing control and an antibacterial agent.

In one embodiment, the wound healing control is selected from the group comprising: a growth factor, for example epidermal growth factor (EGF) or vascular
5 endothelial growth factor (VEGF), fibronectin and vitronectin.

In one embodiment, the control agent is an antibacterial agent.

In one embodiment, the antibacterial agent is selected from the group comprising: an antibiotic, such as gentamicin, an antimicrobial peptide, an antimicrobial polymer and an extracellular vesicle (EV).

10 In one embodiment, the antibacterial agent is an EV, such as an adipose stem cell EV (ASC-EV).

In one embodiment, the antibacterial agent is an antimicrobial peptide, for example selected from the group comprising ENO3, SPARCL1 and V8LQ19L.

In one aspect, there is provided an *in vitro* chronic wound model, method or
15 screening platform substantially as described herein, for example with reference to the drawings.

BRIEF DESCRIPTION OF FIGURES

20 **Figure 1** shows the number of articles published overtime related to (A) skin wound models and (B) non-animal wound models without any animal data.

Figure 2A shows a schematic representation of inoculation of bacteria cellulose (BC) pellicle with *P. aeruginosa* (PA). (i) Pellicle was cut into small pieces to pass through 6mm diameter of the fence-collar-well device. (ii) Pellicle was then autoclaved to make
25 them sterile and dried at RT. (iii) Pellicle placed inside the fence-collar-well device at the centre. (iv) 5% of overnight grown PA strain was inoculated into the BC pellicle for 2 days to allow the bacteria to form biofilms.

Figure 2B shows a flowchart indicating that Biofilm estimation was done by either viable colony counts of PA or by imaging of extracellular polysaccharides (EPS),
30 a major component of bacterial biofilms.

Figure 3 shows images of (A) PA, (B) EF-inoculated and (C) un-infected BC pellicle. Upper panel showed calcofluor staining of EPS and Cellulose and lower panel showed Syto-9-stained bacteria. Scale bar; upper panel, 100 µm. lower panel, 3 µm.

Figure 3D is a graph showing that Crystal violet staining revealed a reduction in
35 biofilm mass following treatments with ENO3, SPARCL1, and gentamicin, n = 3. ANOVA

for multiple comparisons (Dunnett's multiple comparisons test) was applied to determine statistical significance, indicated by * $p < 0.05$; ** $p < 0.01$.

Figure 4 shows a schematic representation of keratinocytes surrounding bacterial biofilms formed on pellicle. Firstly, keratinocytes were seeded from the outlet for 48-72h to have an overlay on cells surrounding the centred wound region. Next, the pellicle containing pre-formed biofilms was placed at the wound region mimicking a chronic wound setting. Migration was monitored upon immediate removal of fence-collar-well devise and imaged hourly for 48h. Post-treatment, each pellicle was sonicated, and the viable counts were obtained by standard plate counts.

Figure 5A shows a table summarising the physicochemical microenvironment characteristic of chronic wounds, and the experimental strategy applied to recreate the wound chronic environment in vitro.

Figure 5B shows a table demonstrating the maintenance of media pH under various conditions. HEPES demonstrated effective buffering capacity, maintaining an alkaline pH even at 1% CO₂.

Figure 5C shows a graph of the gene expression of HIF-1 α as an indicator of hypoxia under various conditions evaluated.

Figure 5D shows a graph showing fluorescence intensity of keratinocytes treated at the indicated conditions each in triplicates from three representative subjects when stained with CellROX™ Deep Red Reagent. Statistical significance was calculated by ANOVA. ** $p < 0.01$ and **** $p < 0.0001$ when compared to the untreated cells.

Figure 5E shows representative images reporting wound dimensions, after 0, 5, and 9 days exposure to listed treatments. Scale bar, 3 μ m.

Figure 5F shows a graph of % wound closure in response to different chronic wound physicochemical conditions. The ImageJ plugin "Wound_healing_size_tool_" was used to measure wound dimensions. Negative wound closure values indicate wound enlargement. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons test, with *** $p < 0.001$ indicating significant differences. The "Untreated" control was used as a reference for calculating statistical significance.

Figure 6A shows representative images illustrating wound closure; in the absence, or the presence of sterile BC pellicle, or PA biofilm-containing pellicle, as annotated. Scale bar, 3 μ m.

Figure 6B shows a graph demonstrating quantification of wound closure. Wound edges were identified and quantitated using the ImageJ plugin "Wound_healing_size_tool_". Statistical analysis was performed using one-way ANOVA

and Dunnett's multiple comparisons test; *** $p < 0.01$, **** $p < 0.001$ relative to untreated control.

Figure 6C shows representative images illustrating closure of keratinocyte monolayers exposed to BC pellicle and peptide V8LQ19L, or gentamicin. Keratinocytes exposed to V8LQ19L remained viable $\leq$ Day 4. Keratinocytes exposed to gentamicin, or without treatment (control), died within 24 hours.

Figure 6D shows a graph of the quantification of the biofilm mass from enumerated bacteria on Day 4 following peptide treatment. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons test, with * $p < 0.05$ indicating significant differences compared to untreated control samples.

Figure 7 shows a schematic representation of chronic wound environment. Wound edges are characterized by elevated levels of ROS, contributing to an alkaline (elevated pH) environment. Poor oxygenation (hypoxia) resulting from poor vascularisation, exacerbates the wound's ability to heal. Persistent microbial colonisation and multispecies biofilms drive sustained inflammation, further complicating the wound environment. The sustained pro-inflammatory state and aberrant microenvironment contribute to cell senescence, and further impede the wound healing process.

EXAMPLES

Example embodiments of the disclosure will be better understood and readily apparent to one of ordinary skill in the art from the following discussions and if applicable, in conjunction with the figures. It should be appreciated that other modifications may be made without deviating from the scope of the invention. Example embodiments are not necessarily mutually exclusive as some may be combined with one or more embodiments to form new exemplary embodiments. The example embodiments should not be construed as limiting the scope of the disclosure.

Example 1 – Materials and Methods

Bacterial strains

Enterococcus faecalis (ATCC 49474, EF), *Pseudomonas aeruginosa* (ATCC 14213, PA) *Komagataeibacter hansenii* 53582 were obtained from The American Type Culture Collection (ATCC).

Visualization of biofilms

To estimate the biofilm mass formed on the pellicles, calcofluor white staining and Syto 9 were used to detect extracellular polysaccharides (EPS) and bacteria respectively. Briefly 0.3 µl of Syto 9 (3.34 mM) (Invitrogen, L7012) was used in 100 µl of reaction mixture for 15 min under dark. Next, Syto 9 stained pellicles were placed on
5 clean glass slides and 1 drop of calcofluor white stain (Sigma-Aldrich 18909) was added followed by one drop of potassium hydroxide. Each stain was put for 1 min, and the excess dyes were removed by paper towels and immediately proceeded for imaging.

Microscopy

Time lapse microscopy was carried out to track the extent of wound regression
10 due to bacterial pellicle loaded with bacterial strains. Image interval of 2 hours and 6 hours was used on the Olympus IX-83 and Nikon TiE microscopes, respectively. The images were auto-stitched to derive an image of an entire well of a 24-well plate. A humidity chamber set to 37°C was used to contain the plates during imaging. To estimate migration potential of keratinocytes, the leading edge of the wound is monitored over
15 time and scored for wound area changes by using 'wound healing size tool plugin' analysed by ImageJ⁴.

Statistical analysis.

Results were represented as means ± SEM. Student's t test was used to determine
20 differences in means between two groups. The p value was calculated using ANOVA for multiple comparisons with corrections and $p < 0.05$ being considered as significant.

Example 2 - Development of bacteria-infected *in-vitro* chronic wound model

(i) Inoculation of bacteria into BC pellicles

25 The BC pellicles were prepared based on⁵ and lyophilization using freeze dryer. BC pellicles were first cut into small pieces (<5mm diameter) and sterilized by autoclaving. Overnight grown PA (5% inoculum) was inoculated into BC pellicles in MHB for 48h to allow bacteria to form biofilms. The pellicles were then washed thrice in PBS to eliminate the non-adhered bacteria. To estimate the amount of PA immobilized, the
30 pellicles were sonicated in a water bath sonicator for 30 min at 15mA, serially diluted and plated to obtain the viable counts. Post-treatment with antibacterial agents, the pellicles were sonicated and plated to estimate the reduction of the viable counts as an effect of treatments.

35 ***(ii) Placing of 'fence-collar-well' device for migration assay.***

Fence-barrier method of leading edge was used to quantify migration as shown in ⁶ with modifications. (A) Fence-collar units were inserted into 24-well tissue culture plates, which formed inner-wells. 40,000 human keratinocytes (HaCat) cells were seeded into each outer-wells and grown for 3 days to have an overlay of cells surrounding the inner wells. (C) To obtain a cell-free area in the inner wells, TrypLE™ Express was added to the inner chambers and the cells were lysed by repeatedly pipetting and washing with PBS.

(iii) Introduction of the BC pellicle containing pre-formed biofilms into the inner well wound site.

BC pellicles containing pre-formed PA biofilms were placed through the inner well into the centred wound region. Next, the fence-collar unit was removed, and the plate was immediately put inside an *Incucyte*® SX5 Live-Cell Analysis Instrument placed inside a 5% CO₂ chamber for 48 hours.

15

(iv) Treatment assessment.

(A) Biofilm disruption potential of EVs were measured by sonicating the BC pellicles to obtain viable counts post-treatment. (B) To estimate migration potential of keratinocytes, the leading edge of the wound is monitored over time and scored for wound area changes by using 'wound healing size tool plugin' ⁴ analysed by ImageJ.

20

Example 3 - Development of the wound model incorporating additional chronic wound characteristics

The present inventors further developed a chronic wound model that encompasses features like alkaline pH, hypoxic conditions, inflammation, and senescent cells. An alkaline pH was achieved by maintaining 1% CO₂ instead of the typical 5%. A hypoxia-mimetic agent, CoCl₂ (20μM), was employed. Etoposide (20μM) was introduced to simulate heightened inflammation and senescence. To represent a comprehensive chronic wound environment, all these conditions were combined.

30

Example 4 - Absence of bacteria-infected *in-vitro* chronic wound model.

Studies (published articles) involving usage of different skin models have emerged in the last 10 years (Figure 1A). Only in the last 3-5 years, very few examples of 2D/3D infected skin wound models and biofilm-infected wound models are cited (Figure 4B). However, a suitable *in-vitro* chronic wound model wherein the presence of

35

bacterial biofilms at the wound site surrounded by skin cells has yet to be reported. The present inventors found no evidence of a wound model platform wherein, dual potency of bacteria killing, and wound healing could be simultaneously investigated.

5 **Example 5 - Evidence of growing bacterial biofilms formed on BC pellicles.**

 The biggest challenge of generating a chronic wound model was to contain the bacteria at the wound site as bacteria can easily swarm, grow massively, deplete nutrients causing rapid acidification, and ultimately kill the keratinocytes by apoptosis within hours. The present inventors made use of BC pellicles obtained from
10 *Komagataeibacter* as shown in ^{5,8}. Next, overnight grown PA was inoculated into the sterile pellicle followed by placement inside collar-fence-well device as described in the *Method* section and depicted in **(Figure 2A)**.

 The present inventors first checked if PA could form biofilms on the BC pellicles by two approaches. In the first approach, biofilms were quantified by sonicating the pellicle and enumerating the viable bacteria by standard plate count **(Figure 2B)**. PA
15 exhibited growing biofilms on the pellicle which was evident from the increase in the viable counts from 3.4×10^4 to 5×10^4 within 24 hours **(Table 1)**. In presence of antimicrobial peptides (AMPs), ENO3, SPARCL1 and antibiotic gentamicin treatments, previously shown to disrupt biofilms (WO2023/219566), decrease in viable counts of PA
20 was observed compared to PBS treatment suggesting biofilm disintegration **(Table 1)**.

Table 1. Viable counts of immobilized PA on the BC pellicle and efficacy of treatment assessments

Conditions	PA-CFU
Seeding density	21,500
Adherent bacteria (after 2 Days)	3.4×10^4
Effects of Treatments (24h)	
PBS	5×10^4
ENO3 (0.2 nM)	$(9.1 \pm 1.8) \times 10^3$
SPARCL1 (0.134 nM)	$(13 \pm 3.1) \times 10^3$
Gentamycin (1.3 μ M)	2.2×10^3

25 **Example 6 - Evidence of EPS validating formation of biofilms by bacteria on BC pellicles.**

In their second approach, the present inventors imaged the pellicle containing EF and PA biofilms by using Syto9 calcofluor staining as stated in Example 1 (**Figure 3**). Biofilms and bacteria were stained as described in the Example 1. No crosstalk or bleed-through was observed between calcofluor (excitation, 347 nm; emission, 300-412) and Syto 9 (excitation, 488 nm; emission, 525 nm). PA showed infiltration into BC matrix (**Figure 3A**). Denser, heterogeneous surface with larger ribbons coated with EPS confirm the presence of both PA and EF biofilms (**Figures 3A, B**). For EF, dense growth observed on BC matrix edges unlike PA, which exhibited uniform distribution (**Figures 3A, B**). Huge ribbon-like structures rather than networks were seen in un-infected pellicle suggesting folding (**Figure 3C**). Consistent with these findings, crystal violet staining of PA-colonized BC samples showed a decrease in biofilm mass in response to the same treatments, underscoring their antibiofilm activities (**Figure 3D**).

These findings confirm that the biofilms formed on the BC pellicles actively develop and are responsive to treatment with antimicrobial proteins. This suggests that BC samples incorporating PA biofilm offer a novel approach to evaluate the efficacy of antibiofilm agents when evaluating rapidly proliferating microbial species.

Example 7 - Implementation of additional wound healing features

Chronic wounds are complex and multifaceted, characterized by more than just the presence of bacteria. They also manifest a distinct alkaline pH⁹, conditions of hypoxia¹⁰, inflammation¹¹, and the presence of senescent cells^{12,13}. The present inventors next set out to emulate these characteristics in our existing model (**Figure 5A**). To maintain an alkaline pH of 8.5 throughout the 48-hour experiment, HEPES was added to the media containing DMEM, which was adjusted to pH 8.5 using 0.1N NaOH under 1% CO₂/95% air conditions (**Figure 5B**). The present inventors then utilized cobalt chloride (CoCl₂, 20uM) to simulate the hypoxic conditions commonly observed in these wounds^{14,15}. Induced hypoxia was confirmed by the upregulated expression of Hypoxia-Inducible Factor (HIF-1α) (**Figure 5C**). Etoposide was incorporated to represent the presence of senescent cells and to elevate oxidative stress levels, mirroring the highly inflamed environment of chronic wounds¹⁶. Reactive oxygen species (ROS) levels, measured using CellROX reagent, confirmed increased oxidative stress in response to etoposide (**Figure 5D**).

Thus, through careful optimization of these physicochemical parameters, both independently and in combination, the present inventors created an in vitro model that closely mimics the complex and inflamed nature of chronic wounds (**Figure 5E**). When

exposed to the combination all four chronic wound physicochemical conditions, the present inventors observed pronounced wound retraction, resulting in significantly enlarged wound areas, recapitulating a hard-to-heal epidermal wound phenotype (Figures 5F). With the introduction of bacterial biofilm-containing pellicles into this model, the present inventors have further enhanced the model's relevance for real-world chronic wound scenarios (Figure 6).

Example 8 - Novel in vitro chronic wound model sustaining coexistence of bacterial biofilms at the wound site and surrounding keratinocytes for concurrent testing of antibiofilm and healing effects.

To recapitulate the microenvironmental conditions more closely in the wound bed of hard to heal wounds, the present inventors introduced PA-infected BC pellicles (Figure 1) at the wound centre, surrounded by an intact monolayer of human keratinocytes (Figure 4). To validate the present inventors' model, they first assessed keratinocyte migration in an 'acute' wound setting: keratinocytes were maintained in optimal pH (7.2), at 37°C in 5% CO₂/95% air, and exposed to: no pellicle, sterile pellicle, and PA biofilm-containing pellicle.

Exposure to sterile pellicles did not hinder wound closure; migration of keratinocytes was equivalent to the no pellicle (aka untreated) control (Figure 6A,B). In contrast, keratinocyte migration in samples exposed to PA biofilm-containing pellicles were significantly attenuated, with evidence of cell death, and increased wound area. The present inventors interpret these data demonstrate the model is effective, providing a platform to access and measure the impact of bacterial biofilm on (Figures 6A,B) epidermal wound closure dynamics — an aspect of epidermal wound healing that is poorly understood.

Having established the ability of their *in vitro* experimental design to provide access to epidermal responses to bacterial biofilms during a wound healing event, the present inventors further evaluated epidermal responses to PA biofilm during wound closure under conditions resembling chronic wounds. When combined with a microenvironment characteristic of chronic wounds, the presence of PA biofilm-containing pellicles precipitated rapid cell detachment and death (within 24 hours) (Figure 6C). Similar levels of cell death were observed in samples exposed to the broad-spectrum antibiotic gentamicin (Figure 6D). Within 24 hours, all cells had died and detached from the vessel surface. Interestingly, samples treated with the novel antibacterial peptide, V8LQ19L (Chakraborty et al, unpublished; PCT/SG2024/050299),

survived, for at least until Day 3, indicating a protective effect, maintaining cell viability (Figure 6D). After 4 days of exposure to V8LQ19L, cell detachment was observed, indicating reduced cell viability (Figure 6D). Bacterial enumeration confirmed that exposure to V8LQ19L resulted in reduced biofilm formation ($P < 0.05$), when compared to samples exposed to gentamicin and untreated control samples.

In summary, the present inventors have developed a simple, yet innovative in vitro model of hard to heal (chronic) epidermal wounds. The simplicity of the platform, compatible with and accessible to existing multi-well format screening technologies and assays, enables simultaneous assessments of bacterial burden and keratinocyte responses to antimicrobial and wound healing interventions. As the pellicles investigated were floating, the biofilm disruption studied was inherently of a floating nature, rather than being surface attached. Floating biofilms play critical roles in various environments, such as aiding *Mycobacterium tuberculosis* persistence in lung cavities¹⁷, enhancing *Vibrio cholerae* colonization in the intestines¹⁸, and contributing to chronic lung infections in cystic fibrosis patients by *Pseudomonas aeruginosa*¹⁹. These floating biofilms are crucial for microbial survival and pathogenicity.

The present inventors' findings underscore the therapeutic potential their novel antibacterial proteins and peptides have for clinical interventions for the management of hard to heal, infected, chronic wounds. To the present inventors' knowledge, this is the only high-content in vitro model that provides simultaneous access to live epidermal wound healing, and to bioburden reduction, positioning it as a pre-clinical a screening tool.

The present inventors foresee that this platform can be further developed. It could, for example, be modified to include multi-species biofilms, and more complex 3D skin models that better mimic the complex physiology and dynamics of human skin (Figure 7).

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10 APPLICATIONS

The present inventors have developed an animal-free chronic wound model that can be used for robust screening of antimicrobials and wound healing agents. In particular, the effects of bacterial burden at the wound site and migration potential of keratinocytes can be co-evaluated in response to any antimicrobials and/or wound
15 healing candidates.

In addition to bacterial burden, chronic wounds exhibit features like alkaline pH, cell senescence, inflammation, and hypoxia. These traits have been successfully incorporated into the model, and by adding bacterial burden, the chronic wound model is able to closely mimic the complexity and detail of an actual chronic wound.

20 Furthermore, the chronic wound models can be utilised in a high-throughput *in-vitro* screening platform for chronic wound treatments. This screening platform can be used for multispecies biofilms, thus enabling diverse experimental setups, making it an attractive choice for robust, high-throughput screening of therapeutics against chronic wound infections. The technology presented here serves as an ideal platform for the
25 study of the chronic wound paradigm and provides a functional system for testing of additional host factors, antimicrobial, and antibiofilm treatments.

CLAIMS

1. An *in vitro* chronic wound model, comprising a wound region surrounded by skin cells, wherein the wound region comprises a bacteria cellulose (BC) pellicle, which has a biofilm comprising one or more species of bacteria.
2. The model according to any preceding claim, further comprising a cell culture vessel which contains the pellicle and the skin cells, for example wherein the skin cells are grown on the surface of the cell culture vessel.
3. The model according to any preceding claim, further comprising a device which forms a barrier between the pellicle and the skin cells, thereby preventing the skin cells from growing into the wound region.
4. A method of producing an *in vitro* chronic wound model comprising a wound region surrounded by skin cells, comprising the steps of:
 - a) inoculating a bacterial cellulose (BC) pellicle with one or more species of bacteria so as to form a biofilm;
 - b) growing a plurality of skin cells in a cell culture vessel; and
 - c) introducing the pellicle into the cell culture vessel, thereby forming the wound region.
5. The method according to any preceding claim, wherein the pellicle is sterilized, for example by autoclaving, prior to inoculation with the one or more species of bacteria.
6. The method according to any preceding claim, wherein the pellicle is inoculated with bacteria for at least 48 hours, for example 48, 60 or 72 hours, in particular for 47 hours.
7. The method according to any preceding claim, wherein the pellicle is washed, for example using a biologically compatible buffer such as PBS, to remove any non-adherent bacteria prior to step c).
8. The method according to any preceding claim, further comprising the step of introducing a device which forms a barrier between the pellicle and the skin cells prior to step b), thereby preventing the skin cells from growing into the wound region.
9. The method according to any preceding claim, wherein the skin cells are grown for 2 or more days, such as 2, 3 or 4 days, in particular for 3 days.
10. The method according to any preceding claim, further comprising the step of introducing an agent to prevent the skin cells from growing into the wound region, for example wherein the agent is trypsin or a derivative thereof, such as TrypLE™ Express.

11. The method according to any preceding claim, further comprising the step of removing the device after step c).
12. The model or method according to any preceding claim, wherein the skin cells are
5 derived from a mammalian organism, for example a primate, a domesticated animal or a livestock animal, for example selected from the group comprising a human, a monkey, a dog, a cat, a sheep, a goat, a cow, or a horse, in particular human skin cells.
13. The model or method according to any preceding claim, wherein the skin cells are
10 keratinocytes.
14. The model or method according to any preceding claim, wherein the biofilm
15 comprises only one species of bacteria.
15. The model or method according to any preceding claim, wherein the biofilm
16 comprises multiple species of bacteria, i.e. at least two different species of bacteria.
16. The model or method according to any preceding claim, wherein the one or more
20 species of bacteria are pathogenic bacteria, for example wherein the bacteria are from a bacterial genus selected from the group comprising: *Bacillus*, *Bartonella*, *Bordetella*, *Borrelia*, *Brucella*, *Campylobacter*, *Chlamydia*, *Chlamydomphila*, *Clostridium*, *Corynebacterium*, *Enterococcus*, *Escherichia*, *Francisella*, *Haemophilus*, *Helicobacter*, *Legionella*, *Leptospira*, *Listeria*, *Mycobacterium*,
25 *Mycoplasma*, *Neisseria*, *Pseudomonas*, *Rickettsia*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, *Treponema*, *Ureaplasma*, *Vibrio* and *Yersinia*.
17. The model or method according to any preceding claim, wherein the one or more
30 species of bacteria are selected from the group comprising: *Acinetobacter baumannii*, *Bacillus anthracis*, *Bacillus cerues*, *Bartonella henselae*, *Bartonella quintana*, *Bordetella pertussis*, *Borrelia burgdorferi*, *Borrelia garinii*, *Borrelia afzelii*, *Borrelia recurrentis*, *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella suis*, *Campylobacter jejuni*, *Chlamydia pneumoniae*, *Chlamydia trachomatis*, *Chlamydomphila psittaci*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium tetani*, *Corynebacterium diphtheriae*, *Enterococcus faecalis*,
35 *Enterococcus faecium*, *Enterobacter* spp, *Escherichia coli*, *Francisella tularensis*, *Hemophilus influenzae*, *Helicobacter pylori*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Leptospira interrogans*, *Leptospira santarosai*, *Leptospira weilii*, *Leptospira noguchii*, *listeria monocytogenes*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Mycobacterium ulcerans*, *Mycoplasma pneumoniae*, *Neisseira gonorrhoeae*, *Neisseira meningitidis*, *Pseudomonas aeruginosa*, *Rickettsia rickettsia*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella sonnei*,
40 *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*,
45 *Streptococcus pyogenes*, *Treponema pallidum*, *Ureaplasma urealyticum*, *Vibrio cholerae*, *Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*,

in particular wherein the one or more species of bacteria are selected from the group comprising *Enterococcus faecalis* and *Pseudomonas aeruginosa*.

18. The model or method according to any preceding claim, wherein the cell culture vessel is a tissue culture plate, for example a 6, 12, 24 or 96 well tissue culture plate, in particular a 24-well tissue culture plate.
19. The model or method according to any preceding claim, wherein the device comprises a fence and a collar, for example as shown in Figure 2A.
20. The model or method according to any preceding claim, wherein the device is located in a well of the tissue culture plate, thereby creating an inner and an outer well.
21. The model or method according to any preceding claim, wherein the pellicle is in the inner well and the skin cells are in the outer well.
22. The model or method according to any preceding claim, further comprising one or more additional cell types, such as senescent cells for example induced by introducing Etoposide to the cells.
23. The model or method according to any preceding claim, wherein the model has one or more of the following environments: an alkaline pH environment, for example achieved by introducing CO₂, such as 1% CO₂ into the model; a hypoxic environment, for example achieved by introducing cobalt chloride, such as 20 µM CoCl₂ into the model; and a high oxidative stress environment, for example achieved by introducing Etoposide into the model.
24. A screening platform, such as a high throughput screening platform, comprising one or more *in vitro* chronic wound models according to any one of the preceding claims.
25. A method of assessing the activity of an agent, comprising the steps of:
 - a) exposing an *in vitro* chronic wound model according to any preceding claim to the agent, followed by either one or both of the following steps:
 - b) determining one of more of the following:
 - the extent of migration,
 - the rate of migration, and
 - the delay in migrationof the skin cells into the wound region of the model; and/or
 - c) performing a count of the viable bacteria present in the pellicle.
26. The method according to claim 25, further comprising step d) comparing the migration measurements and/or colony counts with the migration measurements and/or colony counts of an *in vitro* chronic wound model according to any preceding claim that has been exposed to a control agent.

27. The method according to claim 25 or 26, wherein the activity is selected from the group comprising anti-microbial activity, wound healing activity, and both anti-microbial and wound healing activity.
- 5 28. The method according to any one of claims 26 to 27, wherein the agent is selected from the group comprising an anti-microbial agent, a wound healing agent, and an agent with anti-microbial and wound healing properties.
- 10 29. The method according to any one of claims 25 to 28, wherein the extent, rate and/or delay of migration is determined by imaging the model, for example by using time lapse microscopy.
- 15 30. The method according to any one of claims 25 to 29, wherein the extent, rate and/or delay of migration is determined by monitoring the leading edge of the wound over time, for example by determining the % wound closure.
31. The method according to any one of claims 25 to 30, wherein the pellicle is sonicated prior to the viable count.
- 20 32. The method according to any one of claims 25 to 31, wherein the control agent is selected from the group comprising media, a wound healing control and an antibacterial agent.
- 25 33. The method according to claim 32, wherein the wound healing control is selected from the group comprising: a growth factor, for example epidermal growth factor (EGF) or vascular endothelial growth factor (VEGF), fibronectin and vitronectin.
- 30 34. The method according to any one of claims 28 to 33, wherein the antibacterial agent is selected from the group comprising: an antibiotic, such as gentamicin; an antimicrobial peptide, for example selected from the group comprising ENO3, SPARCL1 and V8LQ19L; an antimicrobial polymer; and an extracellular vesicle (EV), such as an adipose stem cell EV (ASC-EV).