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In Vitro and *In Vivo* Biofilm Wound Models and Their Application

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Abstract

Chronic wounds are wounds which are detained in one or more phases of normal wound healing. It is estimated that 1–2 % of the population of developed countries will experience a chronic wound during their lifetime and this number is expected to increase given the growing world population, increase in age, body mass index and associated diseases such as diabetes and cardiovascular diseases. Although several factors contribute to wound healing, presence of bacterial biofilms significantly affects healing and success of wound treatment. This indicates that wound-care therapies should be directed towards targeting biofilms within chronic wounds. Despite this, the role of biofilms in chronic wound pathogenesis and the effect of wound-care therapies against biofilms within wounds are not well understood. In order to address these issues, appropriate biofilm models are necessary. To this end, several model systems mimicking the conditions observed in a biofilm infected chronic wound have been developed. In this review we present an overview of these different *in vitro* and *in vivo* biofilm wound model systems and discuss their advantages and disadvantages.

Keyword

Chronic wounds • Biofilms • *in vitro* wound biofilm models

1 Introduction

Chronic wounds are wounds which are detained in one or more phases of normal wound healing (Lazarus et al. 1994). Diabetic, arterial, venous and pressure ulcers constitute the majority of these wounds. Chronic wounds affect between two and seven million of patients annually with

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treatment costs rising up to several billions of dollars annually (Sen et al. 2009). It has been estimated that 1–2 % of the population of developed countries will experience a chronic wound during their lifetime and this number is expected to increase given the growing world population, increase in age, body mass index and associated diseases such as diabetes and cardiovascular diseases (Gottrup 2004). Although several factors contribute to wound healing, bacterial infections can significantly affect healing and success of wound treatment (Robson 1997; White and Cutting 2006; Wolcott et al. 2010b). The moist environment and the constant supply of nutrients within the wound represent the ideal environment for bacterial growth. These bacteria can come from different exogenous (e.g. soil and water) as well as endogenous (e.g. skin, saliva, urine, faeces) sources. However, the biodiversity is suggested to be relatively low and *Staphylococcus aureus* and *Pseudomonas aeruginosa* seem to predominate in chronic wounds (Bowler 1998; Fazli et al. 2009; Gjodsbol et al. 2006; Kirketerp-Moller et al. 2008; Rao and Lipsky 2007; Rhoads et al. 2012).

Increasing evidence suggest that these bacteria reside within biofilms in these wounds (Bjarnsholt et al. 2008; Burmølle et al. 2010; Church et al. 2006; James et al. 2008). Biofilms are sessile communities characterized by microbial cells that are irreversibly attached to a substratum and/or to each other and are embedded in a self-produced matrix of extracellular polymeric substances and exhibit an altered phenotype compared to planktonic cells (Costerton et al. 1999). Bacteria living in these biofilms are well protected against antimicrobial agents and host defenses and are for that reason extremely difficult to eradicate (Fux et al. 2003; Bjarnsholt et al. 2008). Recent studies have shown that the major reason for the failure of wound treatment and the shift from acute towards a chronic wound is the presence of bacterial biofilms within the wounds (Harrison-Balestra et al. 2003; Bjarnsholt et al. 2008; Davis et al. 2008; Kirketerp-Møller 2008; Kirketerp-Møller and Gottrup 2009). Only 6 % of acute wounds contained biofilms while this was between 60 and 80 % for chronic wounds (James et al. 2008). In addition, in a study of Dowd et al. (2009) only wounds without detectable biofilm showed signs of wound healing. This indicates that wound-care therapies should be directed towards targeting biofilms within chronic wounds. Despite this, the role of biofilms in chronic wound pathogenesis and the effect of wound-care therapies against these biofilms are not well understood. In order to address these issues, appropriate biofilm models are necessary. To this end, several model systems mimicking the conditions observed in a biofilm infected chronic wound have been developed. In this review we present an overview of these different *in vitro* and *in vivo* biofilm wound model systems and discuss their advantages and disadvantages.

2 Static *In Vitro* Wound Models

Different biofilm models have been used to evaluate the effect of antimicrobial agents on biofilms (see Coenye and Nelis 2010 for a general overview of biofilm model systems). These "general purpose models" can be used to evaluate the efficacy of wound care products or to evaluate biofilm formation of wound isolates. However, most of these *in vitro* models do not reflect the micro environmental conditions found in the wound bed. For this reason, several researchers have made specific adaptations to these general static biofilm models trying to better mimic wound-like environments in an easy-to-handle *in vitro* setting. For example, static biofilm models were developed in which biofilms were grown on agar, poloxamer gels or cellulose matrixes placed in petri-dishes (Clutterbuck et al. 2007; Percival et al. 2007; Hammond et al. 2011; Merritt et al. 2011; Kim and Izadjoo 2015) (Table 1). Although poloxamer gels are polysaccharides, bacterial cultures growing on this substrate mimic many of the properties of biofilm-grown bacteria. Similarly, the permeable nature of cellulose disks allows diffusion of nutrients to the bacteria on the disk, just as nutrients are supplied to biofilms in a wound. As such, both set-ups have been used to evaluate

Table 1 Overview of different static *in vitro* chronic wound models

Characteristics	Percival et al. (2007)	Sun et al. (2008)	Werthén et al. (2010)	Kostenko et al. (2010)	Hammond et al. (2011)	Kucera et al. (2014)
Designation	Poloxamer model	LBCW	Collagen wound model	MBEC wound model	Cellulose agar model	Artificial wound bed model
Use of a wound like surface	No	No/Yes	Yes	Yes	No	No
	(Poloxamer gel)	(plastic tip, silicone disk or host-derived matrix)	(Collagen matrix)	(Serum coated pegs)	(Cellulose disks)	(Plastic)
Use of a wound like medium	No	Yes	Yes	No	No	Yes
	(MH-agar)	(Bolton Broth, 50 % bovine plasma, 5 % freeze-thawed lacked horse-blood)	(SWF: 50 % fetal calf serum and 50 % physiological NaCl in 0.1 % Pepton (PW) or a 1:1 TSB-SWF solution)	(TSB)	(LB-agar)	(Bolton Broth, 1 % gelatine, 50 % porcine plasma, 5 % freeze-thawed porcine erythrocytes or Bolton broth +1 % gelatine +1.2 % agar)
Air-liquid interface	Yes	No	Yes	No	Yes	Yes
Flow present	No	No	No	No	No	No
Inoculum	10^5–10^6 CFU	10^4 CFU	10^4–10^5 CFU	10^7 CFU/ml	10^2–10^4 CFU	10^4 CFU
Incubation temp	25–35 °C	37 °C	35–37 °C	37 °C	37 °C	37 °C

the effect of silver containing dressings (Percival et al. 2007, 2011), antibiotic ointments and agents (Clutterbuck et al. 2007; Hammond et al. 2011; Miller et al. 2014) and garlic (Nidadavolu et al. 2012). In addition, Kostenko et al. (2010) evaluated the efficacy of silver containing dressings using an MBEC ("Minimal biofilm eradication concentration") device. This set-up allows a non-destructive transfer of the biofilms into fresh medium. Biofilms in this device grow on pegs attached to the lid of the device which were coated with serum. Although, most of these general batch culture models have the advantage of being simple and allowing high throughput screening in a cost-effective manner and although some adaptations have been made to better reflect a wound environment, none of them convincingly mimics the conditions observed in an *in vivo* wound.

2.1 Lubbock Chronic Wound Biofilm Model and Derived Models

The first chronic wound model that truly attempted to mimic wound like conditions was developed at the medical biofilm research institute in Lubbock (Texas, US) and was therefore named the "Lubbock chronic wound biofilm model (LCWB)" (Table 1) (Sun et al. 2008). This model allowed the rapid (24 h) cultivation of a robust multispecies biofilm in which *P. aeruginosa*, *S. aureus* and *Enterococcus faecalis* are present in roughly equal ratios. These bacteria were chosen since they are often isolated from and co-occur in chronic wounds (Sun et al. 2008; Gjodsbol et al. 2006). However, the LCWB allows growth of several Gram negative and Gram positive bacteria, aerobes as well

as anaerobes (DeLeon et al. 2014; Dalton et al. 2011). An inoculum of 10^4 cells was used to represent a normal microbial load of a wound prior to infection. Biofilms are grown in a medium consisting of a chopped meat-based medium (Bolton broth) with 50 % heparinized bovine plasma and 5 % freeze-thaw laked horse red blood cells. As such the medium presents the major host factors (e.g. damaged tissue, red blood cells and plasma) found in a typical wound bed. A major downside of this model is the fact that biofilms are formed using a plastic tip or silicone disks as a substrate, which does not reflect a wound-like surface (Sun et al. 2008; Brackman et al. 2011). However, it was recently shown that the medium coagulates into a jelly-like mass when a coagulase-positive bacterial species is used (such as *S. aureus*). *S. aureus* secretes staphylocoagulase which binds to prothrombin, forming a complex which converts soluble fibrinogen to insoluble fibrin. As such there is no need for using an artificial surface since a host-derived matrix is formed which can serve as a scaffold to which bacteria can adhere and form biofilms (DeLeon et al. 2014). Another encouraging aspect of this *in vitro* model is the morphological similarity that is being observed, both with the naked eye as well as on electron micrographs, between biofilms grown in the model and biofilms on actual chronic wounds. As such, this model was shown to be a realistic *in vitro* model which is easy to handle and allows rapid growth and maturation of a multispecies biofilm in a cost effective manner. For this reason, the LCWB has been used extensively to study interspecies interactions (Dalton et al. 2011; DeLeon et al. 2014) and to assess the effect of antibiofilm compounds, antimicrobial agents, hydrogels, functionalized gauzes and dressings against both single species biofilms and polymicrobial communities (Garcia-Fernandez et al. 2013; Luna-straffon et al. 2014; Douglas et al. 2014; Sun et al. 2009; Dowd et al. 2009; Brackman et al. 2011).

Since the first publication, several research groups have made adaptations to the LCBW model to address specific needs. The evaluation of the effect is typically based on quantification of the number of biofilm cells by plating or by using quantitative qPCR methods, making it less suitable for screening large amounts of compounds. Recently, the LCWB was modified for high throughput testing to address this need (Brackman et al. 2013). A good correlation was observed between the fluorescence from a fluorescent *S. aureus* strain and the number of biofilm cells present after treatment (Brackman et al. 2013).

The LCWB model is often used to obtain polymicrobial wound-like biofilms which are then transplanted into other *in vitro* and/or *in vivo* models of skin infection. For example, Dalton et al. (2011) successfully transplanted a biofilm cultured in the LCWB model into a murine skin wound to induce *in vivo* formation of wound biofilms. In addition, Kucera et al. (2014) developed an artificial wound bed model for assessment of solid antimicrobial dressings based on the LCBW model. In brief, the biofilm was pre-cultured using the LCBW set-up with some modifications and amendments. These included the addition of gelatin to the wound medium and the use of porcine plasma and freeze-thaw lacked porcine erythrocytes instead of bovine plasma and horse blood. This pre-cultured biofilm was then transferred onto an artificial wound bed. This artificial wound bed consists of a two-layer nutrient medium composed of Bolton Broth supplemented with 1 % gelatin (w/v) and 1.2 % agar (w/v). The use of the artificial wound bed in the model enables to mimic the situation in chronic infected wounds where the biofilm is only in partial contact with the wound dressing. The modified set-up also incorporates an air-liquid interface feature which is usually present in wound biofilms.

2.2 Collagen-Based *In Vitro* Wound Models

In *in vitro* models, biofilms are often formed on solid, artificial surfaces. This makes it difficult to correlate the *in vitro* results with *in vivo* observations, since the full contribution of the surface to biofilm formation and biofilm

persistence is often unknown. In addition, bacteria in wounds are often not attached to well-defined solid surfaces, but instead reside in the wound bed. For this reason, a model system in which sessile bacteria are aggregated in the absence of a solid surface would mimic the conditions in the wound more closely. To address this issue, Werthén et al. (2010) developed an *in vitro* wound model in which biofilms can develop in the presence of simulated wound fluid (containing 50 % fetal calf serum and 50 % physiological saline in 0.1 % peptone) and a matrix of polymerized rat-tail collagen type I but in the absence of a solid surface (Table 1). Both *P. aeruginosa* and *S. aureus* formed aggregates, surrounded by self-produced polysaccharide matrix within the collagen matrix (Werthén et al. 2010). In addition, biofilms formed in this model were structurally similar to biofilms observed *in vivo*, suggesting the presence of a "wound-like" environment (Werthén et al. 2010). The deep penetration of *P. aeruginosa* biofilms and the more surface-oriented biofilms of *S. aureus* observed in this model resembled other *ex vivo* observations (Kirketerp-Moller et al. 2008). For this reason, this model was used to better predict the *in vivo* antimicrobial activity of antibiotics and silver-containing wound-dressings in several studies (Brackman et al. 2011; Hakonen et al. 2014).

3 *In Vitro* Chronic Wound Models with Liquid Flow

Although the above mentioned biofilm models aim to mimic *in vivo* wound-like environments, all of them are based on closed and therefore accumulative batch culture systems. For this reason, some argue that it is unlikely that they will fully represent the true dynamic state of the wound environment. To address this issue several *in vitro* wound models were developed in which a fluid flow is present and/or in which the biofilm is exposed to shear stress (Thorn and Greenman 2009; Lipp et al. 2010; Hill et al. 2010) (Table 2). The *in vitro* flat-bed perfusion model (Thorn and Greenman 2009), developed based on previously described static models (Greenman et al. 2006; Thorn et al. 2007) addresses this issue. This model consists of autoclavable removable cassettes containing microscope slides on which 1 cm^2 cellulose matrices are placed. A hyperdermic needle, linked to a peristaltic pump was used to perfuse growth medium through the removable cassettes. The medium consists of 0.1 % heat-inactivated foetal calf serum (FCS) or 2 % FCS + 0.1 % glucose in phosphate buffered saline depending on whether *P. aeruginosa* or *S. aureus* was used, respectively (Thorn and Greenman 2009). This model can be used to determine the antimicrobial kill kinetic profile of topically applied treatments (Thorn et al. 2009). In addition, a bioluminescent target organism was integrated into the model and shows the feasibility of using light production for real-time monitoring of antimicrobial efficacy (Thorn and Greenman 2009).

Similarly, Lipp et al. (2010) used a colony drip-flow reactor (C/DFR) model to grow *P. aeruginosa* and *S. aureus* biofilms under wound-like conditions. This model was based on characteristics of both the colony biofilm model (Anderl et al. 2000) and the drip-flow reactor (DFR) model (Buckingham-Meyer et al. 2007). In the C/DFR, biofilms are grown on semipermeable membranes which are placed on microscope slides in a DFR apparatus. These membranes are inoculated with approximately 10^4 CFU of a single species (*P. aeruginosa* or *S. aureus*), left for 30 min to allow drying of the inoculum after which medium (10 % TSB) was pumped through the system (5 ml/h/channel) and biofilms were allowed to form for up to 72 h at room temperature (Lipp et al. 2010). Although initially single species biofilms were grown, growth of a polymicrobial biofilm consisting of bacteria with variable oxygen requirements is possible in this model (Woods et al. 2012). Interesting is the fact that growth of a strict anaerobe (*C. perfringens*) occurred in a polymicrobial biofilm with *P. aeruginosa* and *S. aureus* in the C/DFR, without establishing an artificial anaerobic environment (Woods et al. 2012). As such this model was used to evaluate the effect of antimicrobial agents (Agostinho et al. 2011) and

Table 2 Overview of different dynamic *in vitro* chronic wound models

Characteristics	Thorn and Greenman (2009)	Lipp et al. (2010)	Hill et al. (2010)	Ngo et al. (2012)	Terry and Neethirajan (2014)
Designation	Flat-bed perfusion model	C/DFR	CDFF	CDC-TNP model	Microfluidic wound model
Use of a wound like surface	No	No	Unclear	No	Yes
	(Cellulose matrix)	(Absorbant pad)	(not disclosed)	(Borosilicate or Teflon)	(Collagen)
Use of a wound like medium	Yes	No	No	No	No
	(Foetal calf serum (FCS) or 2 % FCS + 0.1 % glucose in PBS)	(10 % TSB)	(TSB or BM)	(TSB or 10 % TSB)	(TSB + 1 % glucose)
Air-liquid interface	Yes	Yes	Partly[a]	Partly[a]	Partly[a]
Flow present	Yes	Yes	Yes	Yes	Yes
	(1 ml/h)	(5 ml/h)	(30 ml/h)	(11.7 ml/min–40 ml/h)	(100–200 µl/h)
Inoculum	10^5 CFU	10^4 CFU	ND	ND	ND
Incubation temp	37 °C	RT (21.5 °C)	37 °C	30–37 °C	35 °C

[a]An air-liquid interface can be present at different stages (e.g. attachment step, biofilm formation step, evaluation of antibacterial therapies), but not during the entire experiment
ND specific number of cells is not disclosed

wound dressings (Lipp et al. 2010) against mono- and three-species biofilms (Woods et al. 2012).

Recently two different models were developed in which biofilms were first grown in a flow-displacement model and then transferred to an adapted novel *in vitro* wound-like set-up (Ngo et al. 2012; Hill et al. 2010; Malic et al. 2011). These two models are the constant depth film fermenter (CDFF) and the Centers for Disease Control (CDC) biofilm reactor. Both models allow the generation of identical, multiple biofilms simultaneously and allow to vary key parameters including nutrient source, temperature, oxygen availability and substrata (Pratten and Wilson 1999). The reproducibility of identical biofilms, the possibility to image biofilms in three-dimensions and in real-time makes these models interesting starting points to make biofilms which can be implemented in other models.

The CDFF consists of a glass chamber housing a rotating stainless steel disc in which a total of 15 sampling pans, each containing five plugs, are placed. The disc is placed at a set depth and rotates while a scraper plate aids in the distribution of medium across the plugs and maintains a constant depth of the biofilm by removing biofilm cells growing higher. Similarly, the CDC reactor consists of a glass vessel with eight removable polypropylene rods, each holding three removable coupons on which biofilms can form (Donlan et al. 2004). These are oriented in such a way that the coupon is perpendicular to the rotating baffle (Buckingham-Meyer et al. 2007). The glass chamber of both models contains both entry and exit ports allowing a continuous flow of fresh medium through the system. Hill et al. (2010) used a constant depth film fermentor (CDFF) to form multispecies biofilms consisting of wound isolates. In brief, biofilms were grown at 37 °C on plug inserts into the CDFF placed at a 400 µm depth. BM (Hill et al. 2010) or BHI (Malic et al. 2011) medium was pumped through the system at a rate of 30 ml/h. After biofilm formation, biofilms were transferred to a moistened dressing in a sterile petridish (Hill et al. 2010). This set-up has been used to evaluate the effect of different antibiotics, commercial dressings and anti-biofilm compounds (Hill et al. 2010). In addition,

this model was further used to evaluate co-aggregation, synergy and antagonism between bacteria isolated from different types of wounds (Hill et al. 2010; Malic et al. 2011). Similarly, a CDC biofilm reactor was used to form single species biofilms which are then placed in an *in vitro* wound model (Ngo et al. 2012; Valente et al. 2014). In brief, biofilms were grown in a CDC biofilm reactor on borosilicate coupons at 30 °C using Trypton soy broth which was supplied at a rate of 11.7 ml/min. After biofilm formation, coupons were taken out of the CDC and embedded into an agar base representing a low nutrient and moist organic wound surface. A major difference with the CDFF set-up was that a constant flow of 1 % TSB at 40 ml/h was provided across the agar surface by an intravenous infusion (Ngo et al. 2012). This model is mainly used to evaluate the effect of negative pressure by itself or in combination with silver impregnated foam dressings on wound biofilms (Ngo et al. 2012; Valente et al. 2014).

4 Microfluidic Wound Models

A major downside of most of the above mentioned methods is the need for relatively large amounts of test-compounds when evaluating their efficacy in these models. Microfluidic-based wound models can overcome this drawback (Zhang et al. 2013). Microfluidic technology is a relatively new field that is already applied to study biofilm growth in a confined space (e.g. mimicking biofilm growth in a blood vessel) (Sato et al. 2014), to study antimicrobial resistance in biofilms by creating dynamic concentration gradients and/or to study spatial and temporal growth of micro-organisms as well as motility and chemotaxis in biofilms (Kim et al. 2012; Halder et al. 2013). Although differences between microfluidic devices exist, the channels are typically 50–500 μm wide, 30–500 μm deep and 1–40 mm in length. In addition, flow rates are usually low (0.1–50 μl/min) (Coenye and Nelis 2010). Recently, a "microfluidic wound model" was described which is easy to use, relatively cheap and small (Terry and Neethirajan 2014) (Table 2). In order to better mimic wound like surfaces, the channels were coated with rat tail collagen type I before bacteria were pumped through the system (Terry and Neethirajan 2014; Chen et al. 2014).

Although microfluidic wound models have several advantages compared to other models (e.g. use of a flow while only small amounts of test product are needed) there is still room for improvement on different other levels (e.g. use of more relevant media, surfaces and mixed biofilms). In this view it is interesting to note that microfluidic co-culture models are being developed in which biofilms can develop in the presence of an epithelial cell monolayer (Kim et al. 2010a, b; Zhang et al. 2013). Recently, Zhang et al. (2013) developed a microfluidic wound-scratch model system to investigate cell migration and proliferation. Although this model was not published in the context of infected wound biofilms, it displays the possibility of upgrading existing models to better emulate the conditions observed in an infected *in vivo* chronic wound.

5 Issues with the *In Vitro* Wound Models

Although all of the above mentioned *in vitro* models address specific aspects of wound biofilms, they all are prone to limitations (Tables 1 and 2). First of all, although some models display flexibility in the use of different bacterial species and/or mixed biofilm communities, most of the *in vitro* wound models only rely on the use of a single bacterial species. As such it is unclear whether these models would allow the incorporation of a biofilm consisting of different bacterial species. Dominant single species biofilm aggregates of *S. aureus* and *P. aeruginosa* are observed in infected chronic wounds and the outcome of wound healing can be correlated with the presence of a specific species. However, infected chronic wounds are often polymicrobial in nature, despite the fact that bacterial diversity is generally low (Robson 1997; Rao and Lipsky 2007; Colsky et al. 1998;

Gjodsbol et al. 2006; Fazli et al. 2009; Rhoads et al. 2012). For this reason, increasing the complexity of the model by adding multiple species could make the model system more relevant.

A second issue is the temperature used. Most of these biofilms are formed and maintained at 37 °C which reflects core body temperature. However, although skin temperature can be different due to variability between persons and body location, temperature of trauma wounds and wound bed temperature of chronic leg ulcers ranges between 25–37 °C and 24–26 °C, respectively (Fierheller and Sibbald 2010; Romanelli et al. 2002). This temperature is significantly lower than what is often used in the different models, which would indicate that conducting experiments at lower temperatures would better reflect the chronic wound bed temperature.

A wide range of different inocula are also being used in these models. These inocula range between 10^2 and 10^8 CFU. It is generally accepted that infected chronic wounds contain more than 10^5 bacteria per gram of tissue (Robson 1997; Bowler 2003). Although it is highly questionable that high bioburden levels are present at the start of infection under proper standard care conditions, models applying these higher inocula might be representative for heavily infected wounds or wounds inflicted under conditions were proper wound-care is not directly possible. In addition, lower inocula can be used for investigating biofilm development from the start of an infection. As such the inoculum used, should depend on the question that needs to be answered and it should be clear whether different inocula can be used in the different model systems.

Thirdly, the surface and media used in some models often do not reflect the nutritional conditions which bacteria would find in wound beds. Surfaces such as glass, silicone and plastics do not resemble the surfaces on which biofilms are formed in real wounds. In addition, although some artificial surfaces (such as poloxamer gels and cellulose disks) do possess some wound-like features, it remains questionable whether these would evoke similar responses in bacterial gene expression, biofilm formation and resistance to therapy as to biofilms grown on biotic surfaces. As such, most of these *in vitro* models do not take into account the role that dermal substrates can play on bacterial attachment, nutrition, biofilm shape and resistance and for this reason these models could be adapted at the level of the surfaces used in order to better mimic wound like conditions. Similarly, general media such as TSB or LB support the growth of a wide variety of microorganisms, but they do not contain many of the components which are present in wound exudates. Specific media such as the simulated wound fluid (Werthén et al. 2010) or media containing plasma, serum, blood cells and/or heparin likely better reflect nutritional conditions observed in wounds. However, to date there is no standardized nutrient medium to replicate wound exudates under *in vitro* conditions and the composition of wound fluid and wound exudates can be highly variable depending individual, type of wounds and wound healing stadium (Trengove et al. 1996, 1999; Cutting 2003; Eming et al. 2010). It thus remains difficult to really define which media would reflect wound conditions best.

Finally, as crucial is the expected geometry of how nutrients are applied to the wound biofilm. Although this might vary depending on the wound type and amount of exudate produced, nutrients generally originate from the host tissue at the bottom of the biofilm, while oxygen is usually supplied from the top of the biofilm at the air-liquid/surface interface. In addition, the physical aspect of a low fluid shear might be important in specific wound types. Although most of the *in vitro* wound model systems take into account one or more of these aspects in order to mimic *in vivo* wounds, none of them take into account all these aspects (Tables 1 and 2).

6 Cell-Based Wound Models

Implementing skin as a substrate for attachment and as the primary source of nutrition for microbial biofilm cells would allow the formation of biofilms under conditions which would more closely resemble the *in vivo* situation. For this

reason, several more advanced cell-based wound models were developed in which porcine skin explants (Yang et al. 2013; Phillips et al. 2013; Wolcott et al. 2010a), two-dimensional cell monolayers or three-dimensional tissue-engineered human skin equivalents (TE-HSE) (Haisma et al. 2013; Charles et al. 2009) were used as a substrate for biofilm development.

Given the fact that pig skin and human skin have striking similarities in structure (Summerfield et al. 2014), cell-based wound models using porcine skin explants have been used to study molecular characteristics of biofilms attaching to skin (Yang et al. 2013), assess the efficacy of antimicrobial agents and antimicrobial wound care dressings against *P. aeruginosa* and *S. aureus* biofilms and assess the effect of negative pressure wound therapy with instillation of antimicrobial solutions against *P. aeruginosa* biofilms (Phillips et al. 2010, 2013). A main disadvantage is that significant differences still exist between human and animal skin at the level of immunological responses (Summerfield et al. 2014). Despite this, human explants have rarely been used since it would be difficult to standardize and reproduce results obtained in such models. The development of reconstituted human tissue models using two-dimensional cell monolayers or three-dimensional tissue-engineered human skin equivalents would overcome this issue. Although monolayer cultured cells are often used, such studies do not accurately reflect the behavior, pathophysiology, or microenvironment of skin *in vivo* (Welss et al. 2004). Cells in monolayer culture are in isolation and for this reason do not take into account that bacteria invade and interact with different cell types in a complex three-dimensional solid structure. For this reason, three-dimensional systems would better mimic *in vivo* infections. Tissue-engineered human skin equivalents (HSE) are three-dimensional systems that mimic the native skin to a high degree (Welss et al. 2004). Although different HSE are described in literature, they are typically generated by culturing primary keratinocytes and dermal fibroblasts at the air-liquid interface of cell-free matrices (e.g. filters, collagen gels or decellularized dermal scaffolds such as de-epidermized dermis). The cells will proliferate, migrate and differentiate during peridermal development resulting in skin equivalents that usually contain all layers of the native epidermis and/ or dermis (El Ghalbzouri et al. 2004, 2008; Charles et al. 2009; Welss et al. 2004; Torkian et al. 2004). In addition, several HSE are commercially available. Epiderm-FT (MatTek, MA, US) is a multilayered highly differentiated skin model consisting of human-derived keratinocytes and fibroblasts in cell culture inserts. Apligraf is a tissue engineered skin equivalent which consists of a lower dermal layer (collagen and human fibroblasts) and an upper epidermal layer (human keratinocytes which can differentiate). In addition, reconstructed human epidermis (RHE, Skinethic, Lyon, France) consists of normal human keratinocytes cultured on an inert polycarbonate filter at the air-liquid interface, in a chemically defined medium. The HSE is typically wounded using a biopsy punch or a device heated or cooled with boiling water or liquid nitrogen, respectively, prior to infection (El Ghalbzouri et al. 2004; Haisma et al. 2013; Shepherd et al. 2009). Others have demonstrated that bacteria can colonize HSE and trigger the expression of pro-inflammatory cytokines/chemokines by the underlying cells (Holland et al. 2008, 2009; De Breij et al. 2012; Haisma et al. 2013; Kirker et al. 2009, 2012; Charles et al. 2009). In addition, HSE wound models were used to assess the antimicrobial activity of different agents and plasma against bacterial biofilms under wound like conditions (Haisma et al. 2013; Shepherd et al. 2009; Brackman et al. 2011).

Recently, Bellas et al. (2012) developed a full-thickness skin equivalent which included epidermis, dermis, and hypodermis. This model would serve as a more physiological relevant system that would likely sustain physiological function for more extended time periods in ways that would permit both acute, short-term, and chronic, long-term studies of skin development and pathogenesis. In addition, the morphology and organization of the tri-layer skin model

would allow secretion of appropriate levels of cytokines and mimic the full spectrum of biological functions of skin. The cell-based models have the advantage that they are histologically similar to human skin and thereby provide a controlled environment similar to the one encountered in *in vivo* wounds. However, unlike human skin, these usually do not contain Langerhans' cells, macrophages, lymphocytes or other structures such as blood cells, hair follicles or sweat glands.

7 *In Vivo* Wound Model Systems

To address the above mentioned issues, several *in vivo* wound models were developed, each with their own strengths and weaknesses (Seth et al. 2012). These animal models are needed since it is virtually impossible to study the development of chronic wound in humans. This is due to ethical concerns, but also due to the fact that the chronic wound is often already present when patients arrive in the clinic. In addition, when these wounds are investigated, this will only be observational thereby lacking the experimental and causative data necessary to fully investigate the role of biofilms and interplay with therapeutically agents (Seth et al. 2012).

One of the first studied *in vivo* models of wound infections relied on the use of *Drosophila melanogaster* (reviewed by Apidianakis and Rahme 2009). A wound infection in the cuticular epithelium and underlying muscle is established in this model by using a thoracic or abdominal pin prick which was dipped in a bacterial suspension. As such, this model was used to study host responses to wound infection by different microbes. Despite being often used, the translation of results obtained in an invertebrate pin-prick wound system to what could be expected in human wounds is questionable. For this reason, mostly vertebrate animals such as mice, rats, pigs and rabbits are used in *in vivo* wound model systems (Table 3). Next to the type and breed of animal used, these models mainly differ in the mechanisms by which wounds are inflicted, how wounds (and infection) is being maintained during the experiment, on the inoculum size and whether or not different bacterial species were shown to be capable of infecting the host under the given circumstances.

Akiyama et al. (1996) described biofilm formation of *S. aureus* in incisional wounds of mice and this model was later on used to evaluate topical treatment on biofilm susceptibility (Akiyama et al. 2002). Similarly, Rumbaugh et al. (1999) and later on Rashid et al. (2000) examined the role of different genes (including quorum sensing genes) on *P. aeruginosa* virulence in a burn wound mouse infection model. However, the effect of biofilm infection on the global wound healing process or host responses was not assessed. Similarly, several other murine infection models are published in which wounds are caused by thermal injury (Trøstrup et al. 2013; Nichols et al. 2013). Although these models can be useful to study burn wound infections, they do not always represent conditions found in chronic wounds which not originated from burns. For this reason several other models have focused on inflicting wounds by other manners such as biopsy punch (Thompson et al. 2014; Schierle et al. 2009; Zhao et al. 2010; Petreaca et al. 2012; Gurjala et al. 2011), surgical incision (Ermolaeva et al. 2011; Asada et al. 2012; Watters et al. 2014) or by means of sanding (Roche et al. 2012a, b) or pressure (Nakagami et al. 2008). Besides inflicting a wound, maintaining a biofilm infection within these models for a certain amount of time remains challenging. For this reason several models rely on specific preconditioned animals (e.g. mutant breeds or induction of specific pathogenesis such as diabetes), the pre-formation of the biofilm under *in vitro* conditions before the biofilm is applied to the wound bed and/or placement of dressing materials to maintain a moist environment (Table 3). Most of the rodent models also ignore the fact that contracture should be minimized in these models. By minimizing contractures, e.g. by placement of silicone rings around the wound bed, wounds are allowed to heal by new tissue ingrowth, more akin to human wounds, as opposed to myofibroblast-mediated

Table 3 Overview of different *in vivo* chronic wound models

Animals	Wound type	Wound location	Wound maintenance	inoculum	Single or mixed species biofilm	Reference
Murine models						
Ddy mice	Cut wounds	Back	–[a]	3.6×10^6 CFU/ml	Single *(S. aureus)*	Akiyama et al. (1996), (2002)
Swiss Webster mice	Thermal injury (90 °C water) (15 % t.b.i.)	Back	–[a]	10^2 CFU	Single *(P. aeruginosa)*	Rumbaugh et al. (1999), Rashid et al. (2000)
Swiss Webster mice	Surgical excision wound (1.5 cm^2)	Dorsal	Opsite dressing	10^4 CFU	Single *(P. aeruginosa* and *S. aureus)*	Watters et al. (2014), Turner et al. (2014)
						Wolcott et al. (2010a), Gawande et al. (2014)
BalB/c mice	Thermal injury (hot air) (6 % t.b.i.)	Back	Seaweed alginate beads	10^7 CFU/ml	Single *(P. aeruginosa)*	Trøstrup et al. (2013))
C57BL/6 J mice	Thermal injury (10 % t.b.i.) and abrasion injury	Dorsal	Abrasion injury prior to infection	10^6 CFU	Single *(P. aeruginosa)*	Nichols et al. 2013
Adult male C57Bl6/J mice	Excisional punch wounds	Back	Silicone rings and covered with Tegaderm	Pre-formed biofilm[b]	Single (*S. aureus* and *S. epidermidis)*	Schierle et al. (2009)
Male SWR/J and male TH mice	Full-thickness dermal wounds (ND)	Dorsal	Silicon splints and tegaderm dressing	10^6 CFU	Single (*S. aureus*)	Nguyen et al. (2013)
Db/db mice	Full-thickness punch wounds	Dorsal	Dressing occlusion	Pre-formed biofilm[b]	Single *(P. aeruginosa)*	Zhao et al. (2010)
BALB/c	Biopsy punch	Dorsal	Tegaderm dressing	5×10^4 CFU	Single *(A. baumannii)*	Thompson et al. (2014)
BALB/c mice	Full thickness wound (ND)	Back	Gauze patch	5×10^7 CFU	Single *(S. aureus)*	Simonetti et al. 2008
LIGHT−/− mice	Biopsy punch	Dorsal	tegaderm	10^8 CFU/ml	Single (*S. epidermidis*)	Petreaca et al. (2012), Dhal et al. (2014)
Mice (type not disclosed)	Wounded by sanding	Back	Moistened bandage	2×10^7 CFU	Single (*S. aureus*)	Roche et al. (2012a)
Adult male Sprague	Surgical incision	Nape (back of the neck)	Cotton pellets	10^8 CFU/ml	Mixed (*P. aeruginosa* and *S. aureus*)	Ermolaeva et al. (2011)
Dawley rats						

(continued)

Table 3 (continued)

Animals	Wound type	Wound location	Wound maintenance	inoculum	Single or mixed species biofilm	Reference
Adult male Sprague	Scissors incision	Flank region	Tegaderm dressing	2×10^9 CFU	Single (*P. aeruginosa*)	Asada et al. (2012)
Dawley rats						
male Wister rats	Pressure-related ischemic wounds	lateroabdominal and dorsal regions	–[a]	10^5 CFU	Single (*P. aeruginosa*)	Nakagami et al. (2008)
Pig models						
Commercially raised, specific pathogen-free, female	Full thickness trephine (2 cm) wounds	Back	Gauze pad and tegaderm dressing	10^7–10^8 CFU	Single (*S. aureus*)	Roche et al. (2012b)
Yorkshire-cross pigs						
Young, female, specific pathogen-free pigs	Wounded using a modified electrokeratome set	Back and side	polyurethane film dressing (tegaderm) and self-adherent bandages	10^6–10^7 CFU/mL	Single and mixed (*P. aeruginosa* and *S. aureus*)	Davis et al. (2001, 2007, 2008), Pechter et al. (2012), Pastar et al. (2013), Nusbaum et al. (2012)
Rabbit models						
New Zealand white rabbits	Full-thickness dermal punch wounds	Ventral side of each ear	Tegaderm dressing	10^6 CFU	Single and mixed (*K. pneumoniae*, *P. aeruginosa* and *S. aureus)*	Gurjala et al. (2011), Seth et al. (2012), Chen et al. (2014), Leung et al. 2014

[a]No specific wound-maintenance strategy mentioned
[b]Pre-formed biofilm. Specific inoculum number not disclosed
ND specific mechanism not disclosed

contraction of the loose rodent skin (Schierle et al. 2009; Nguyen et al. 2013). Additionally, only a limited amount of models study the infection for a longer period of time (Thompson et al. 2014; Roy et al. 2014). Although the use of mice and rats have some advantages over the use of larger animals such as pigs (e.g. ease-of-use, space-limitations, economical and ethical concerns), pigs are preferred for wound healing studies due to higher similarities between porcine and human skin and due to the scale at which wounds can be introduced (Sullivan et al. 2001; Summerfield et al. 2014). In addition, with respect to the translational value, the use of pigs as preclinical model for wound studies is recommended (Gordillo et al. 2013). Recently, an *in vivo* biofilm wound infection model was developed in rabbits (Gurjala et al. 2011). This model was based on the rabbit dermal ulcer model, which is an FDA-recognized model of wound healing which has been used for over two decades (Mustoe et al. 1991; Chen et al. 1999; Said et al. 2005; Mogford et al. 2009). In this model, full-thickness, circular punch-wounds are made in the ears of New Zealand White rabbits down to cartilage, affording a number of important advantages. For example, in contrast to partial-thickness wounds, this removal of dermis more closely models the dermal-loss seen in human chronic wounds. Additionally, the majority of human wounds heal through epithelialization and granulation, in contrast to the contracture-based healing seen in mice (Schierle et al. 2009). The underlying cartilage of the rabbit ear serves as a natural splint, preventing healing by contracture, and thus allowing for accurate quantification of epithelial and granulation tissue formation from the periphery of the wound. Moreover, multiple identical wounds can be made in one animal with contralateral controls, creating a standardized and high-throughput wound model. In contrast to other published models where pre-formed *in vitro* biofilm is directly applied to wounds, these wounds are inoculated with planktonic, free-floating bacteria which more closely represents the seeding mechanism of human chronic wounds, with the wound bed itself playing a critical role in the transformation of bacteria into the biofilm state (Schultz et al. 2004; Cierny and DiPasquale 2006). Although different *in vivo* models exist, the clinical relevance of these models is still being argued (Seth et al. 2012). These aspects should be addressed in the future.

8 Concluding Remarks

Investigating wound infections and development of novel therapeutic agents targeting these types of infections require the existence of appropriate models. As discussed in this review, several *in vitro* and *in vivo* wound model systems have been described, each with their specific strengths and weaknesses and addressing different aspects of wound biofilms. As such, researchers should select a model by measuring out these differences against the questions that they are hoping to answer using these models. However, due to the complexity of wound healing, extrapolation of results from *in vitro* biofilm studies to the clinic will always remain challenging. Only animal models can take into account factors such as interplay of immune reponses and wound bed components. In addition, *in vivo* animal models are necessary, since it is virtually impossible to study the development of chronic wound in humans. For this reason, there is a wide consensus that there is a high need for not only conducting these experiments, but also for a further development and improvement of the existing models both *in vitro* as well as *in vivo*. These modifications, including the introduction of polymicrobial biofilms, more relevant media and surfaces, would possibly lead to models which are truly capable of evaluating therapies under *in vitro* and *in vivo* settings. In addition, better models would eventually lead to studies on biochemical pathways (e.g. by use of mutants), host response to infection and on the interplay between different therapeutically agents and the biofilms which would better reflect reality. This would ultimately improve our understanding of why chronic wounds develop and why they are being maintained and altogether these insights

could possibly lead to better therapies addressing the issue of chronic wound infections in the clinic in the future.

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References

Agostinho A, Hartman A, Lipp C, Parker A, Stewart P, James G (2011) An in vitro model for the growth and analysis of chronic wound MRSA biofilm. J Appl Microbiol 111:1275–1282

Akiyama H, Kanzaki H, Tada J, Arata J (1996) *Staphylococcus aureus* infection on cut wounds in the mouse skin: experimental Staphylococcal botryomycosis. J Dermatol Sci 11:234

Akiyama H, Huh WK, Yamasaki O, Oono T, Iwatsuki K (2002) Confocal laser scanning microscopic observation of glycocalyx production by *Staphylococcus aureus* in mouse skin: does *S. aureus* generally produce a biofilm on damaged skin? Br J Dermatol 147:879

Anderl JN, Franklin MJ, Stewart PS (2000) Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. Antimicrob Agents Chemother 44(7):1818–1824

Apidianakis Y, Rahme LG (2009) *Drosophila melanogaster* as a model host for studying *Pseudomonas aeruginosa* infection. Nat Protoc 4:1285

Asada M, Nakagami G, Minematsu T, Nagase T, Akase T, Huang L, Yoshimura K, Sanada H (2012) Novel models for bacterial colonization and infection of full-thickness wounds in rats. Wound Repair Regen 20:601–610

Bellas E, Seiberg M, Garlick J, Kaplan DL (2012) *In vitro* 3D full-thickness skin-equivalent tissue model using silk and collagen biomaterials. Macromol Biosci 12 (12):1627–1636

Bjarnsholt T, Kirketerp-Moller K, Jensen PO, Madsen KG, Phipps R, Krogfelt K, Høiby N, Givskov M (2008) Why chronic wounds will not heal: a novel hypothesis. Wound Repair Regen 16:2–10

Bowler PG (1998) The anaerobic and aerobic microbiology of wounds: a review. Wounds 10(6):170–178

Bowler PG (2003) The 10(5) bacterial growth guideline: reassessing its clinical relevance in wound healing. Ostomy Wound Manage 49(1):44–53

Brackman G, Cos P, Maes L, Nelis HJ, Coenye T (2011) Quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics *in vitro* and *in vivo*. Antimicrob Agents Chemother 55:2655–2661

Brackman G, Demeyer L, Nelis H, Coenye T (2013) Biofilm inhibitory and biofilm eradicating activity of wound care products against *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms in an *in vitro* chronic wound model. J Appl Microbiol 114:1833–1842

Buckingham-Meyer K, Goeres DM, Hamilton MA (2007) Comparative evaluation of biofilm disinfectant efficacy tests. J Microbiol Methods 70(2):236–244

Burmølle M, Thomsen TR, Fazli M, Dige I, Christensen L, Homøe P, Tvede M, Nyvad B, Tolker-Nielsen T, Givskov M, Moser C, Kirketerp-Møller K, Johansen HK, Høiby N, Jensen PØ, Sørensen SJ, Bjarnsholt T (2010) Biofilms in chronic infections – a matter of opportunity – monospecies biofilms in multispecies infections. FEMS Immunol Med Microbiol 59(3):324–336

Charles CA, Ricotti CA, Davis SC, Mertz PM, Kirsner RS (2009) Use of tissue-engineered skin to study in vitro biofilm development. Dermatol Surg 35(9):1334–1341

Chen EA, Zhao L, Bamat M, von Borstel R, Mustoe T (1999) Acceleration of wound healing with topically applied deoxyribonucleosides. Arch Surg 134:520–525

Chen P, Seth AK, Abercrombie JJ, Mustoe TA, Leung KP (2014) Activity of imipenem against *Klebsiella pneumoniae* biofilms *in vitro* and *in vivo*. Antimicrob Agents Chemother 58(2):1208–1213

Church D, Elsayed S, Reid O, Winston B, Lindsay R (2006) Burn wound infections. Clin Microbiol Rev 19:403–434

Cierny G III, DiPasquale D (2006) Treatment of chronic infection. J Am Acad Orthop Surg 14:S105–S110

Clutterbuck AL, Cochrane CA, Dolman J, Percival SL (2007) Evaluating antibiotics for use in medicine using a poloxamer biofilm model. Ann Clin Microbiol Antimicrob 6:2

Coenye T, Nelis HJ (2010) *In vitro* and *in vivo* model systems to study microbial biofilm formation. J Microbiol Methods 83(2010):89–105

Colsky AS, Kirsner RS, Kerdel FA (1998) Microbiologic evaluation of cutaneous wounds in hospitalized dermatology patients. Ostomy Wound Manage 44:40–46

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322

Cutting KF (2003) Wound exudate: composition and function. Br J Community Nurs 8(9 Suppl):suppl 4–9

Dalton T, Dowd SE, Wolcott RD, Sun Y, Watters C, Griswold JA, Rumbaugh KP (2011) An *in vivo* polymicrobial biofilm wound infection model to study interspecies interactions. PLoS One 6:e27317

Davis SC, Eaglstein WH, Cazzaniga AL, Mertz PM (2001) An octyl- 2-cyanoacrylate formulation speeds healing of partialthickness wounds. Dermatol Surg 27:783–788

Davis SC, Ricotti C, Cazzaniga A, Welsh E, Eaglstein WH, Mertz PM (2007) Microscopic and physiologic evidence for biofilm-associated wound colonization in vivo. Wound Repair Regen. 2008 Jan-Feb;16(1):23-9. doi: 10.1111/j.1524-475X.2007.00303.x.

Davis SC, Ricotti C, Cazzaniga A, Welsh E, Eaglstein WH, Mertz PM (2008) Microscopic and physiologic evidence for biofilm-associated wound colonization in vivo. Wound Repair Regen 16(1):23–9. doi:10.1111/j.1524-475X.2007.00303.x

De Breij A, Haisma EM, Rietveld M, El Ghalbzouri A, van den Broek PJ, Dijkshoorn L, Nibbering PH (2012) Three-dimensional human skin equivalent as a tool to study *Acinetobacter baumannii* colonization. Antimicrob Agents Chemother 56(5):2459–2464

DeLeon S, Clinton A, Fowler H, Everett J, Horswill AR, Rumbaugh KP (2014) Synergistic interactions of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in an *in vitro* wound model. Infect Immun 82 (11):4718–4728

Dhall S, Do D, Garcia M, Wijesinghe DS, Brandon A, Kim J, Sanchez A, Lyubovitsky J, Gallagher S, Nothnagel EA, Chalfant CE, Patel RP, Schiller N, Martins-Green M (2014) A novel model of chronic wounds: importance of redox imbalance and biofilm-forming bacteria for establishment of chronicity. PLoS One 9(10), e109848. doi:10.1371/journal.pone.0109848

Donlan RM, Piede JA, Heyes CD, Sanii L, Murga R, Edmonds P, El-Sayed I, El-Sayed MA (2004) Model system for growing and quantifying Streptococcus pneumonia biofilms in situ and in real time. Appl Environ Microbiol 708:4980–4988

Douglas EL, Piwowarczyk W, Pamula E, Liskova J, Schaubroeck D, Leeuwenburgh SCG, Brackman G, Balcaen L, Detsch R, Cholewa-Kowalska K, Vanhaecke F, Cornelissen R, Coenye T, Boccaccini A, Dubruel P (2014) Injectable self-gelling composites for bone tissue engineering based on gellan gum hydrogel enriched with different bioglasses. Biomed Mater 9(4):045014

Dowd SE, Sun Y, Smith E, Kennedy JP, Jones CE, Wolcott R (2009) Effects of biofilm treatments on the multi-species Lubbock chronic wound biofilm model. J Wound Care 18(508):510–512

El Ghalbzouri A, Hensbergen P, Gibbs S, Kempenaar J, van der Schors R, Ponec M (2004) Fibroblasts facilitate re-epithelialization in wounded human skin equivalents. Lab Investig 84:102–112

El Ghalbzouri A, Siamari R, Willemze R, Ponec M (2008) Leiden reconstructed human epidermal model as a tool for the evaluation of the skin corrosion and irritation potential according to the ECVAM guidelines. Toxicol In Vitro 22:1311–1320

Eming SA, Koch M, Krieger A, Brachvogel B, Kreft S, Bruckner-Tuderman L, Krieg T, Shannon JD, Fox JW (2010) Differential proteomic analysis distinguishes tissue repair biomarker signatures in wound exudates obtained from normal healing and chronic wounds. J Proteome Res 9(9):4758–4766

Ermolaeva SA, Varfolomeev AF, Chernukha MY, Yurov DS, Vasiliev MM, Kaminskaya AA, Moisenovich MM, Romanova JM, Murashev AN, Selezneva II, Shimizu T, Sysolyatina EV, Shaginyan IA, Petrov OF, Mayevsky EI, Fortov VE, Morfill GE, Naroditsky BS, Gintsburg AL (2011) Bactericidal effects of non-thermal argon plasma *in vitro*, in biofilms and in the animal model of infected wounds. J Med Microbiol 60(1):75–83

Fazli M, Bjarnsholt T, Kirketerp-Moller K, Jorgensen B, Andersen AS, Krogfelt KA, Givskov M, Tolker-Nielsen T (2009) Nonrandom distribution of Pseudomonas aeruginosa and Staphylococcus aureus in chronic wounds. J Clin Microbiol 47:4084–4089

Fierheller M, Sibbald RG (2010) A clinical investigation into the relationship between increased periwound skin temperature and local wound infection in patients with chronic leg ulcers. Adv Skin Wound Care 23 (8):369–379

Fux CA, Stoodley P, Hall-Stoodley L, Costerton JW (2003) Bacterial biofilms: a diagnostic and therapeutic challenge. Expert Rev Anti Infect Ther 1:667–683

Garcia-Fernandez MJ, Brackman G, Coenye T, Concheiro A, Alvarez-Lorenzo C (2013) Antiseptic cyclodextrin-functionalized hydrogels and gauzes for loading and delivery of benzalkonium chloride. Biofouling 29:261–271

Gjodsbol K, Christensen JJ, Karlsmark T, Jorgensen B, Klein BM, Krogfelt KA (2006) Multiple bacterial species reside in chronic wounds: a longitudinal study. Int Wound J 3:225–231

Gordillo GM, Bernatchez SF, Diegelmann R, Di Pietro LA, Eriksson E, Hinz B, Hopf HW, Kirsner R, Liu P, Parnell LK, Sandusky GE, Sen CK, Tomic-Canic M, Volk SW, Baird A (2013) Preclinical models of wound healing: is man the model? Proceedings of the Wound Healing Society Symposium. AdvWound Care (New Rochelle) 2:1–4

Gottrup F (2004) A specialized wound healing center concept: importance of a multidisciplinary department structure and surgical treatment facilities in the treatment of chronic wounds. Am J Surg 187:38S–43S

Greenman J, Thorn RM, Saad S, Austin AJ (2006) In vitro diffusion bed, 3-day repeat challenge 'capacity' test for antimicrobial wound dressings. Int Wound J 3:322–329

Gurjala AN, Geringer MR, Seth AK, Hong SJ, Smeltzer MS, Galiano RD, Leung KP, Mustoe TA (2011) Development of a novel, highly quantitative *in vivo* model for the study of biofilm-impaired cutaneous wound healing. Wound Repair Regen 19:400

Gawande PV, Clinton AP, LoVetri K, Yakandawala N, Rumbaugh KP, Madhyastha S (2014 Mar 5) Antibiofilm efficacy of DispersinB(®) wound spray used in combination with a silver wound dressing. Microbiol Insights 7:9–13

Haisma EM, Rietveld MH, de Breij A, van Dissel JT, El Ghalbzouri A, Nibbering PH (2013) Inflammatory and antimicrobial responses to methicillin- resistant Staphylococcus aureus in an in vitro wound infection model. PLoS One 8(12):e82800

Hakonen B, Lönnberg LK, Larkö E, Blom K (2014) A novel qualitative and quantitative biofilm assay based on 3D soft tissue. Int J Biomat. Article ID 768136

Halder P, Nasabi M, Lopez FJ, Jayasuriya N, Bhattacharya S, Deighton M, Mitchell A, Bhuiyan MA (2013) A novel approach to determine the

efficacy of patterned surfaces for biofouling control in relation to its microfluidic environment. Biofouling 2013(29):697–713

Hammond AA, Miller KG, Kruczek CJ, Dertien J, Colmer-Hamood JA, Griswold JA, Horswill AR, Hamood AN (2011) An in vitro biofilm model to examine the effect of antibiotic ointments on biofilms produced by burn wound bacterial isolates. Burns 37 (2):312–321

Harrison-Balestra C, Cazzaniga AL, Davis SC, Mertz PM (2003) A wound-isolated *Pseudomonas aeruginosa* grows a biofilm in vitro within 10 hours and is visualized by light microscopy. Dermatol Surg 29:631–635

Hill KE, Malic S, McKee R, Rennison T, Keith GH, Williams DW, Thomas DW (2010) An in vitro model of chronic wound biofilms to test wound dressings and assess antimicrobial susceptibilities. J Antimicrob Chemother 65:1195–1206

Holland DB, Bojar RA, Jeremy AH, Ingham E, Holland KT (2008) Microbial colonization of an in vitro model of a tissue engineered human skin equivalent--a novel approach. FEMS Microbiol Lett 279:110–115

Holland DB, Bojar RA, Farrar MD, Holland KT (2009) Differential innate immune responses of a living skin equivalent model colonized by Staphylococcus epidermidis or Staphylococcus aureus. FEMS Microbiol Lett 290:149–155

James GA, Swogger E, Wolcott R, Pulcini E, Secor P, Sestrich J, Costerton JW, Stewart PS (2008) Biofilms in chronic wounds. Wound Repair Regen 16:37–44

Kim H, Izadjoo MJ (2015) Antibiofilm efficacy evaluation of a bioelectric dressing in mono- and multi-species biofilms. J Wound Care 24(Suppl 2):S10–S14

Kim J, Hegde M, Jayaraman A (2010a) Microfluidic co-culture of epithelial cells and bacteria for investigating soluble signal-mediated interactions. J Vis Exp 38:1749

Kim J, Hegde M, Jayaraman A (2010b) Co-culture of epithelial cells and bacteria for investigating host–pathogen interactions. Lab Chip 10:43–50

Kim J, Park HD, Chung S (2012) Microfluidic approaches to bacterial biofilm formation. Molecules 17 (9818):9834

Kirker KR, Secor PR, James GA, Fleckman P, Olerud JE, Stewart PS (2009) Loss of viability and induction of apoptosis in human keratinocytes exposed to Staphylococcus aureus biofilms in vitro. Wound Repair Regen 17:690–699

Kirker KR, James GA, Fleckman P, Olerud JE, Stewart PS (2012) Differential effects of planktonic and biofilm MRSA on human fibroblasts. Wound Repair Regen 20(2):253–261

Kirketerp-Møller K, Gottrup F (2009) [Bacterial biofilm in chronic wounds]. Ugeskr Laeger 171:1097

Kirketerp-Moller K, Jensen PO, Fazli M, Madsen KG, Pedersen J, Moser C, Tolker-Nielsen T, Hoiby N, Givskov M, Bjarnsholt T (2008) Distribution, organization, and ecology of bacteria in chronic wounds. J Clin Microbiol 46(8):2717–2722

Kostenko V, Lyczak J, Turner K, Martinuzzi RJ (2010) Impact of silver-containing wound dressings on bacterial biofilm viability and susceptibility to antibiotics during prolonged treatment. Antimicrob Agents Chemother 54(12):5120–5131

Kucera J, Sojka M, Pavlik V, Szuszkiewicz K, Velebny V, Klein P (2014) Multispecies biofilm in an artificial wound bed—a novel model for in vitro assessment of solid antimicrobial dressings. J Microbiol Methods 103(2014):18–24

Lazarus GS, Cooper DM, Knighton DR, Margolis DJ, Pecoraro RE, Rodeheaver G, Robson MC (1994) Definitions and guidelines for assessment of wounds and evaluation of healing. Arch Dermatol 130:489–493

Leung KP, D'Arpa P, Seth AK, Geringer MR, Jett M, Xu W, Hong SJ, Galiano RD, Chen T, Mustoe TA (2014) Dermal wound transcriptomic responses to Infection with Pseudomonas aeruginosa versus Klebsiella pneumoniae in a rabbit ear wound model. BMC Clin Pathol 14:20. doi:10.1186/1472-6890-14-20

Lipp C, Kirker K, Agostinho A, James G, Stewart P (2010) Testing wound dressings using an in vitro wound model. J Wound Care 19:220–226

Luna-Straffon MA, Contreras-García A, Brackman G, Coenye T, Concheiro A, Alvarez-Lorenzo C, Bucio E (2014) Wound debridement and antibiofilm properties of gamma-ray DMAEMA-grafted cotton gauzes. Cellulose J 21:3767–3779

Malic S, Hill KE, Playle R, Thomas DW, Williams DW (2011) In vitro interaction of chronic wound bacteria in biofilms. J Wound Care 20(12):569–570

Merritt JH, Kadouri DE, O'Toole GA (2011) Growing and analyzing static biofilms. Curr Protoc Microbiol 22:1–8

Miller KG, Tran PL, Haley CL, Kruzek C, Colmer-Hamood JA, Myntti M, Hamood AN (2014) Next science wound gel technology, a novel agent that inhibits biofilm development by gram-positive and gram-negative wound pathogens. Antimicrob Agents Chemother 58(6):3060–3072

Mogford JE, Tawil B, Jia S, Mustoe TA (2009) Fibrin sealant combined with fibroblasts and platelet derived growth factor enhance wound healing in excisional wounds. Wound Repair Regen 17:405–410

Mustoe TA, Pierce GF, Morishima C, Deuel TF (1991) Growth factor-induced acceleration of tissue repair through direct and inductive activities in a rabbit dermal ulcer model. J Clin Invest 87:694–703

Nakagami G, Sanada H, Sugama J, Morohoshi T, Ikeda T, Ohta Y (2008) Detection of Pseudomonas aeruginosa quorum sensing signals in an infected ischemic wound: an experimental study in rats. Wound Repair Regen 16 (1):30–36. doi:10.1111/j.1524-475X.2007.00329.x

Ngo QD, Vickery K, Deva AK (2012) The effect of topical negative pressure on wound biofilms using an in vitro wound model. Wound Repair Regen 20(1):83–90

Nguyen KT, Seth AK, Hong SJ, Geringer MR, Xie P, Leung KP, Mustoe TA, Galiano RD (2013) Deficient cytokine expression and neutrophil oxidative burst

contribute to impaired cutaneous wound healing in diabetic, biofilm-containing chronic wounds. Wound Repair Regen 21(6):833–841

Nichols DP, Caceres S, Caverly L, Fratelli C, Kim SH, Malcolm K, Poch KR, Saavedra M, Solomon G, Taylor-Cousar J, Moskowitz S, Nick JA (2013) Effects of azithromycin in Pseudomonas aeruginosa burn wound infection. J Surg Res 183(2):767–776

Nidadavolu P, Amor W, Tran PL, Dertien J, Colmer-Hamood JA, Hamood AN (2012) Garlic ointment inhibits biofilm formation by bacterial pathogens from burn wounds. J Med Microbiol 61(Pt 5):662–671

Nusbaum AG, Gil J, Rippy MK, Warne B, Valdes J, Claro A, Davis SC (2012 Aug) Effective method to remove wound bacteria: comparison of various debridement modalities in an in vivo porcine model. J Surg Res 176 (2):701–7. doi:10.1016/j.jss.2011.11.1040

Pastar I, Nusbaum AG, Gil J, Patel SB, Chen J, Valdes J, Stojadinovic O, Plano LR, Tomic-Canic M, Davis SC (2013) Interactions of methicillin resistant Staphylococcus aureus USA300 and Pseudomonas aeruginosa in polymicrobial wound infection. PLoS One 8(2), e56846. doi:10.1371/journal.pone.0056846

Percival SL, Bowler PG, Dolman J (2007) Antimicrobial activity of silver-containing dressings on wound microorganisms using an in vitro biofilm model. Int Wound J 4(2):186–191

Percival SL, Thomas JG, Slone W, Linton S, Corum L, Okel T (2011) The efficacy of silver dressings and antibiotics on MRSA and MSSA isolated from burn patients. Wound Repair Regen 19(6):767–774

Petreaca ML, Do D, Dhall S, McLelland D, Serafino A, Lyubovitsky J, Schiller N, Martins-Green MM (2012) Deletion of a tumor necrosis superfamily gene in mice leads to impaired healing that mimics chronic wounds in humans. Wound Repair Regen 20(3):353–366

Pechter PM, Gil J, Valdes J, Tomic-Canic M, Pastar I, Stojadinovic O, Kirsner RS, Davis SC (2012) Keratin dressings speed epithelialization of deep partial-thickness wounds. Wound Repair Regen. 2012 Mar-Apr;20 (2):236-42. doi: 10.1111/j.1524-475X.2012.00768.x.

Phillips PL, Yang Q, Sampson EM, Schultz GS (2010) Effects of antimicrobial agents on an in vitro biofilm model of skin wounds. In: Sen CK (ed) Advances in wound care. Wound Healing Society Yearbook Publication. Mary Ann Liebert Inc. Publishers, New Rochelle, pp 299–304.

Phillips P, Yang Q, Sampson E, Progulske-Fox A, Antonelli P, Shouquang J, Schultz G (2013) Development of a novel *ex vivo* porcine skin explant model for the assessment of mature bacterial biofilms. Wound Repair Regen 21:704–714

Pratten J, Wilson M (1999) Antimicrobial susceptibility and composition of microcosm dental plaques supplemented with sucrose. Antimicrob Agents Chemother 43:1595–1599

Rao N, Lipsky BA (2007) Optimising antimicrobial therapy in diabetic foot infections. Drugs 67:195–214

Rashid MH, Rumbaugh K, Passador L, Davies DG, Hamood AN, Iglewski BH, Kornberg A (2000) Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 97:9636

Rhoads DD, Wolcott RD, Sun Y, Dowd SE (2012) Comparison of culture and molecular identification of bacteria in chronic wounds. Int J Mol Sci 13 (3):2535–2550

Robson MC (1997) Wound infection. A failure of wound healing caused by an imbalance of bacteria. Surg Clin North Am 77:637–650

Roche ED, Renick PJ, Tetens SP, Carson DL (2012a) A model for evaluating topical antimicrobial efficacy against methicillin-resistant Staphylococcus aureus biofilms in superficial murine wounds. Antimicrob Agents Chemother 56(8):4508–4510

Roche ED, Renick PJ, Tetens SP, Ramsay SJ, Daniels EQ, Carson DL (2012b) Increasing the presence of biofilm and healing delay in a porcine model of MRSA-infected wounds. Wound Repair Regen 20 (4):537–543

Romanelli M, Gaggio G, Coluccia M, Rizzello F, Piaggesi A (2002) Technological advances in wound bed measurements. Wounds 14(2):58–66

Roy S, Elgharably H, Sinha M, Ganesh K, Chaney S, Mann E, Miller C, Khanna S, Bergdall VK, Powell HM, Cook CH, Gordillo GM, Wozniak DJ, Sen CK (2014) Mixed-species biofilm compromises wound healing by disrupting epidermal barrier function. J Pathol 233(4):331–343

Rumbaugh KP, Griswold JA, Iglewski BH, Hamood AN (1999) Contribution of quorum sensing to the virulence of *Pseudomonas aeruginosa* in burn wound infections. Infect Immun 67:5854–5862

Said HK, Hijjawi J, Roy N, Mogford J, Mustoe T (2005) Transdermal sustained-delivery oxygen improves epithelial healing in a rabbit ear wound model. Arch Surg 140:998–1004

Sato K, Sasaki N, Svahn HA, Sato K (2014) Microfluidics for nano-pathophysiology. Adv Drug Deliv Rev 74:115–121

Schierle CF, De la Garza M, Mustoe TA, Galiano RD (2009) Staphylococcal biofilms impair wound healing by delaying reepithelialization in a murine cutaneous wound model. Wound Repair Regen 17:354

Schultz GS, Barillo DJ, Mozingo DW, Chin GA, Wound Bed Advisory Board Members (2004) Wound bed preparation and a brief history of TIME. Int Wound J 1:19–32

Sen CK, Gordillo GM, Roy S, Kirsner R, Lambert L, Hunt TK, Gottrup F, Gurtner GC, Longaker MT (2009) Human skin wounds: a major and snowballing threat to public health and the economy. Wound Repair Regen 17:763–771. Seth et al. 2012.

Seth AK, Geringer MR, Hong SJ, Leung KP, Mustoe TA, Galiano RD (2012) *In vivo* modeling of biofilm-infected wounds: a review. J Surg Res 178(1):330–338

Shepherd J, Douglas I, Rimmer S, Swanson L, MacNeil S (2009) Development of three-dimensional tissue engineered models of bacterial infected human skin wounds. Tissue Eng Part C Methods 15:475–484

Simonetti O, Cirioni O, Ghiselli R, Goteri G, Scalise A, Orlando F, Silvestri C, Riva A, Saba V, Madanahally KD, Offidani A, Balaban N, Scalise G, Giacometti A (2008) RNAIII-Inhibiting peptide enhances healing of wounds infected with methicillin-resistant Staphyloccus aureus. Antimicrob Agents Chemother 52:2205

Sullivan TP, Eaglstein WH, Davis SC, Mertz P (2001) The pig as a model for human wound healing. Wound Repair Regen 9(2):66–76

Summerfield A, Meurens F, Ricklin ME (2014) The immunology of the porcine skin and its value as a model for human skin. Mol Immunol. http://dx.doi.org/10.1016/j.molimm.2014.10.023

Sun Y, Dowd SE, Smith E, Rhoads DD, Wolcott RD (2008) In vitro multispecies Lubbock chronic wound biofilm model. Wound Repair Regen 16:805–813

Sun Y, Smith E, Wolcott R, Dowd SE (2009) Propagation of anaerobic bacteria within an aerobic multi-species chronic wound biofilm model. J Wound Care 18:426–431

Terry J, Neethirajan S (2014) A novel microfluidic wound model for testing antimicrobial agents against Staphylococcus pseudintermedius biofilms. J Nanobiotechnol 12:1

Thompson MG, Black CC, Pavlicek RL, Honnold CL, Wise MC, Alamneh YA, Moon JK, Kessler JL, Si Y, Williams R, Yildirim S, Kirkup BC Jr, Green RK, Hall ER, Palys TJ, Zurawski DV (2014) Validation of a novel murine wound model of Acinetobacter baumannii infection. Antimicrob Agents Chemother 58(3):1332–1342

Thorn RM, Greenman J (2009) A novel in vitro flat-bed perfusion biofilm model for determining the potential antimicrobial efficacy of topical wound treatments. J Appl Microbiol 107:2070–2079

Thorn RM, Nelson SM, Greenman J (2007) Use of a bioluminescent Pseudomonas aeruginosa strain within an in vitro microbiological system, as a model of wound infection, to assess the antimicrobial efficacy of wound dressings by monitoring light production. Antimicrob Agents Chemother 51:3217–3224

Thorn RM, Austin AJ, Greenman J, Wilkins JP, Davis PJ (2009) In vitro comparison of antimicrobial activity of iodine and silver dressings against biofilms. J Wound Care 18(8):343–346

Torkian BA, Yeh AT, Engel R, Sun CH, Tromberg BJ, Wong BJF (2004) Modeling aberrant wound healing using tissue-engineered skin constructs and multiphoton microscopy. Arch Facial Plast Surg 6:180–187

Trengove NJ, Langton SR, Stacey MC (1996) Biochemical analysis of wound fluid from nonhealing and healing chronic leg ulcers. Wound Repair Regen 4 (2):234–239

Trengove NJ, Stacey MC, MacAuley S, Bennett N, Gibson J, Burslem F, Murphy G, Schultz G (1999) Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors. Wound Repair Regen 7(6):442–452

Trøstrup H, Thomsen K, Christophersen LJ, Hougen HP, Bjarnsholt T, Jensen PØ, Kirkby N, Calum H, Høiby N, Moser C (2013) Pseudomonas aeruginosa biofilm aggravates skin inflammatory response in BALB/c mice in a novel chronic wound model. Wound Repair Regen 21(2):292–299

Turner KH, Everett J, Trivedi U, Rumbaugh KP, Whiteley M (2014 Jul 24) Requirements for Pseudomonas aeruginosa acute burn and chronic surgical wound infection. PLoS Genet 10(7), e1004518. doi:10.1371/journal.pgen.1004518

Valente PM, Deva A, Ngo Q, Vickery K (2014) The increased killing of biofilms in vitro by combining topical silver dressings with topical negative pressure in chronic wounds. Int Wound J. doi:10.1111/iwj.12248

Watters C, Everett JA, Haley C, Clinton A, Rumbaugh KP (2014) Insulin treatment modulates the host immune system to enhance Pseudomonas aeruginosa wound biofilms. Infect Immun 82(1):92–100

Welss T, Basketter DA, Schröder KR (2004) In vitro skin irritation: facts and future. State of the art review of mechanisms and models. Toxicol In Vitro 18 (3):231–243

Werthén M, Henriksson L, Jensen PØ, Sternberg C, Givskov M, Bjarnsholt T (2010) An in vitro model of bacterial infections in wounds and other soft tissues. APMIS 118(2):156–164

White RJ, Cutting KF (2006) Critical colonization--the concept under scrutiny. Ostomy Wound Manage 52 (11):50–56

Wolcott RD, Rumbaugh KP, James G, Schultz G, Phillips P, Yang Q, Watters C, Stewart PS, Dowd SE (2010a) Biofilm maturity studies indicate sharp debridement opens a time- dependent therapeutic window. J Wound Care 19(8):320–328

Wolcott RD, Rhoads DD, Bennett ME, Wolcott BM, Gogokhia L, Costerton JW, Dowd SE (2010b) Chronic wounds and the medical biofilm paradigm. J Wound Care 19:45–53

Woods J, Boegli L, Kirker KR, Agostinho AM, Durch AM, Delancey Pulcini E, Stewart PS, James GA (2012) Development and application of a polymicrobial, in vitro, wound biofilm model. J Appl Microbiol 112(5):998–1006

Yang Q, Phillips PL, Sampson EM, Progulske-Fox A, Jin S, Antonelli P, Schultz GS (2013) Development of a novel *ex vivo* porcine skin explant model for the assessment of mature bacterial biofilms. Wound Repair Regen 21(5):704–714

Zhang M, Li H, Ma H, Qin J (2013) A simple microfluidic strategy for cell migration assay in an in vitro wound-healing model. Wound Repair Regen 2013(21):897–903

Zhao G, Hochwalt PC, Usui ML, Underwood RA, Singh PK, James GA, Stewart PS, Fleckman P, Olerud JE (2010) Delayed wound healing in diabetic (db/db) mice with Pseudomonas aeruginosa biofilm challenge: a model for the study of chronic wounds. Wound Repair Regen 18:467