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Electronic Supplementary Information (ESI)

Non-invasive, 3D printed, colourimetric, early wound-infection indicator

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S1 Examples of sensors used to detect biomarkers in chronic wounds

Smart wound dressings that are capable of reporting early stages of microbial growth through monitoring biological cues, such as uric acid, pH, bacteria, oxygen, and temperature, have been developed and recently reviewed.[1, 2] Some examples of the sensors used to detect these biomarkers in chronic wounds are given in the table below.

Table S.1: Examples of sensors used to detect biomarkers in chronic wounds.

Sensor	Biomarker	Method	Ref
Carbon fibre sensor	Uric Acid	Electrochemical	3
Poly-tryptophan carbon fiber pH sensor	pH	Voltammetry	4, 5
Flexible hydrogel pH sensor	pH	Fluorescent spectroscopy/Image processing	6
Intelligent hydrogel dressing	Bacteria (*S. aureus, P. aeruginosa, E. coli, E. faecalis*)	Electrochemical/Fluorescent spectroscopy	7
Oxygen bandage sensor	Oxygen	Bio-electrical	8
Flexible Pt thermistor	Temperature	Electrical	9

S2 Experimental

2.1 Materials

Unless otherwise stated, all chemicals and solvents were purchased from Sigma-Aldrich in the highest purity available. All gases used were purchased from BOC at the highest purity.

Pseudomonas aeruginosa (*P. aeruginosa,* NCIMB 10548), a common wound pathogen,[10] was purchased as a lyophilised powder from the National Collection of Industrial, Food and Marine Bacteria (NCIMB) and maintained at -80 °C in 15v/v% glycerol.

Enterococcus faecium (E. faecium, NCTC 7379), *Acinetobacter baumannii (A. baumannii,* NCTC 13304), *Streptococcus pyogenes (S. pyogenes,* NCTC 8306), *Candida albicans (C. albicans,* NCYC 610) *and Staphylococcus aureus (S. aureus,* NCTC 10788) were purchased from the National Collective of type cultures. Organic, stillborn piglets were ethically sourced from Agri-food and Biosciences Institute (AFBI) research farm (Hillsborough, Northern Ireland) and immediately stored at -20 °C until further use. The wound dressing used in this work was occlusive,[11] 3M™ Tegaderm™ transparent film dressings 1624W (6×7 cm) which was purchased from Amazon UK.[12]

Simulated wound fluid (SWF) was prepared using a recipe reported elsewhere[13] which, briefly involved mixing 50% foetal bovine serum (FBS) (Gibco, Thermo Fisher, Warrington, UK) with 50% physiological NaCl (Merck, Dorset, UK) in 0.1% bacteriological peptone (Oxoid, Basingstoke, UK). Luria-Bertani broth (LBB) was purchased from Invitrogen (Thermo Fisher, Warrington, UK). Pseudomonas selective Cetrimide agar was purchased from Merck (Gillingham, UK). For other pathogens, a lab made Luria-Bertani Agar (LBA) was used. Phosphate-buffered saline (PBS) was purchased from Merck (Dorset, UK). All solutions for microbiological work were prepared fresh on-the day and autoclaved prior to use.

2.2 Methods

2.2.1 Preparation of the carbon dioxide (CO_2) sensitive indicator

In this work, Xylenol Blue (XB) was chosen as the colour-based CO_2 sensor dye, due to its moderate pK_a (= 9.52),[14] which rendered it particularly sensitive towards CO_2, striking colour change (blue in its deprotonated form, XB^-, and yellow in its protonated form, HXB) and low cost. The CO_2-sensitive pigment was made by coating the XB on hydrophilic fumed silica particles (Aerosil 130 V, Evonik Industries (Essen, Germany), particle size ~20 nm). Briefly, 0.2 g of XB were dissolved in 100 ml ethanol, and 3.2 ml of tetrabutyl ammonium hydroxide (TBAH, 1 M in ethanol) and 2 g hydrophilic fumed silica were added the to the mixture. After stirring for 2 hours, the solvent was removed by rotary evaporation, leaving a bright blue pigment comprising particles of silica coated with XB and the TBAH. This blue-coloured, CO_2-sensitive pigment was then used to make a CO_2 indicator-containing filament for 3D printing by mixing 1 g of the pigment with 19 g of low-density polyethylene (LDPE) powder (PW Hall UK Ltd., Glasgow, UK, melt flow index, melt flow index (MFI) = 20) and extruding as a filament using a Rondol Microlab twin-screw extruder (Rondol, Nancy, France), thermostatted at 90 °C at the feed zone and with a temperature profile which gradually increased along its length to 140 °C at the exit (i.e. die) zone; the extruder screw speed was always run at 80 rpm. LDPE was used to encapsulate the CO_2 sensitive pigment particles because of its low cost, high gas permeability, biological and chemical inertness, and low processing temperature. The latter feature is particularly important, since if processed above 180 °C, as is usually required for many other polymers, such as polypropylene, that would otherwise be suitable the dye in the pigment degrades. The extruded 2 mm diameter filament was withdrawn at a rate of 0.9 m min^{-1} and chopped up, using an inline pelletiser, into 3 mm long pellets to create an initial masterbatch of pellets. This pelleted masterbatch was then fed through the extruder 3 more times to ensure a homogeneous distribution of the pigment throughout each pellet.

Instead of extruding the masterbatch match pellet into plastic film,[15, 16] in this communication, we have taken this step further. For the first time, CO_2 indicator was 3D printed, as it is an ideal method for the low cost scaled up production of plastic materials.[17] Hence, the final homogenised masterbatch pellets were then fed into a filament maker (model: the Composer 350, 3devo B.V.,

Utrecht, Netherlands) to generate a filament of the XB/TBAH/LDPE based CO_2 indicator, with a diameter of 1.75 ± 0.05 mm, as required for use with the 3D printer. The CO_2 indicator-loaded filament was then 3D printed at 135 °C as a 50 µm thick, 12x12 mm square indicator film on the glass platform of the printer heated to 80 °C. The 3D printer used was a ZMorph25 VX Full Set FDM (ZMorph, Wrocław, Poland), fitted with 0.3 mm nozzle.

2.2.2. *Preparation of damaged porcine skin samples*

The porcine skin was defrosted before use and washed with deionised water. Then the skin was depilated, and any excess connective tissue or muscle removed to provide a smooth, even surface and rinsed once more in deionised water before allowing to dry. A cork-borer (14 mm diameter) and surgical scalpel was used to excise each circular skin explant. Score marks were made in a criss-cross fashion on the skin using a surgical scalpel to create a 'wounded' environment for pathogens to grow and washed with 70% ethanol for 20 minutes to disinfect the skin. Finally, each explant was dipped in 100% ethanol and flamed to remove any remaining ethanol and provide a dry, disinfected surface for inoculation. Photographic images of the porcine skin in the various stages of its treatment are illustrated below in Figure S2.1.

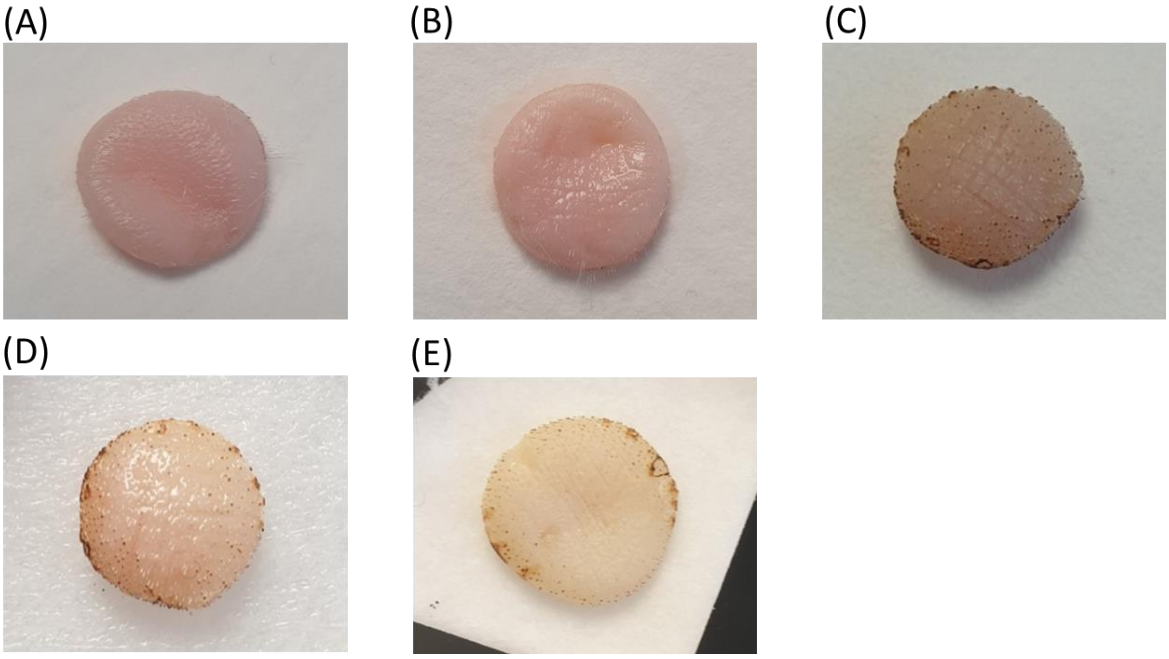

Figure S2.1. Photographic images of **(A)** Depilated porcine skin. **(B)** Skin scored with a scalpel and disinfected with 70% ethanol for 20 min. **(C)** Skin dipped in 100% ethanol and flamed. **(D)** Skin inoculated with 20 µL of suspension. **(E)** Skin following 24 h incubation at 30 °C.

2.2.3 Preparation of inocula

P. aeruginosa was resuscitated from frozen stocks into LBB and grown at 37 °C overnight. The overnight culture was centrifuged (5 min, 5000 x g) and resuspended in SWF and adjusted to an optical density of 0.15 at 600 nm corresponding to 10^8 CFU ml^{-1} as confirmed by viable count. Serial one in ten dilutions were performed in SWF to achieve subsequent suspensions of 10^7, 10^6, 10^5, 10^4, and 10^3 CFU ml^{-1}.

2.2.4 Preparation of smart wound dressing on inoculated 'wounded' porcine skin samples

Sheets of 50 µm thick PET were cut to size (8 x 10 cm) and disinfected by wiping with a 70% ethanol aqueous solution. Sterile absorbent pads, 47 mm diameter (Merck Millipore Ltd., UK), were aseptically cut into quarters and placed on the PET sheet. Each quarter was rendered damp with 500 µL of sterile water to prevent the porcine skin from drying out. A sample of the porcine skin (200 mg, 14 mm in diameter) was placed onto the absorbent pad and inoculated with 20 µL of either 0, 10^3, 10^4, 10^5, 10^6,

10^7, or 10^8 CFU ml^{-1} *P. aeruginosa* in SWF. Four thin sheets of sterile lint were cut to size (2×2 cm) and placed over the inoculated 'wounded' porcine skin. The inoculated porcine skin/lint dressing indicator was then sealed to the underlying PET film using a Tegaderm™ transparent film (48 μm thick) occlusive dressing (6×7 cm) to create a dressed inoculated, 'wounded' porcine skin sample with CO_2 indicator, a schematic illustration of which is illustrated in Figure S2.2.

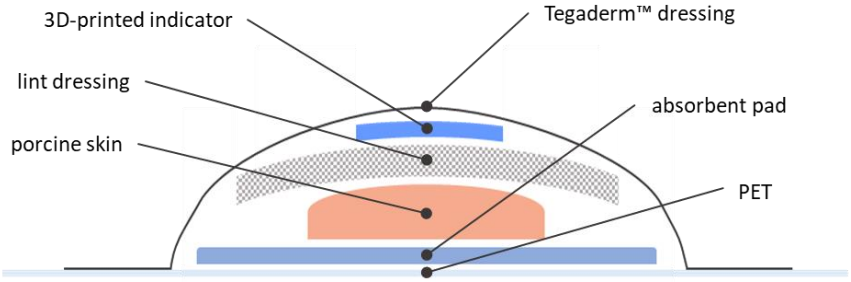

Figure S2.2: Schematic of the 'smart' wound dressing with a sample of inoculated, damaged porcine skin placed on a 50 μm film of clear PET. The dressing itself comprised a damp absorbent pad, on top of which were placed, a piece of inoculated damaged porcine skin, followed by a lint dressing and the 3D-printed indicator, all sealed in with a Tegaderm™ occlusive dressing.

Each of these 'smart' wound dressings was incubated at 30 °C and the colour of the CO_2 indicator monitored periodically using a digital camera over a 30 h period. A recent study into neuropathic diabetic and non-diabetic foot ulcers[18] found that the *'mean wound temperatures at baseline were largely below 33 °C (mean 30.90 °C) independent of a diagnosis of diabetes or not and all in noninfected wounds'* and goes on to suggest a new temperature range of 28 – 32 °C. Thus, in all this work the mean of this temperature range, i.e. 30 °C, was used as the incubation temperature.

2.2.5 Monitoring bacterial growth

In separate experiments where the focus was to determine the increase in microbial load/bioburden with incubation time, the same 'smart' wound dressing with a sample of inoculated, damaged porcine skin, as described in section 1.2.4, was used but this time incubated at 30 °C for either 0, 6, 12, 24 or 30 h, after which the level of viable bacteria on the porcine skin sample (typical tissue mass = 0.2 g) was determined (units: CFU g (tissue)$^{-1}$) using the Miles and Misra plate count method.[19] In the plate method, at each timepoint, i.e. 0, 6, 12, 24 or 30 h, the porcine skin sample and lint were harvested from the dressing and placed in 2 ml PBS. Samples were vortexed (Vortex-Genie 2, Scientific Industries, New York, US) for 30 s, sonicated (Elmasonic S 30, Elma, Bedford, UK) for 5 min and vortexed again for 30 s to remove any adherent bacteria. Serial dilutions were performed in PBS.[19] Dilutions were plated onto Cetrimide agar and incubated overnight at 37 °C and the counts combined with knowledge of the dilutions employed were then used to determine the bioburden on the damaged porcine skin in units of (units: CFU g^{-1}). Cetrimide agar is an established selective media for the growth of *P. aeruginosa*, and so was chosen to facilitate the selective, accurate plate counting of *P. aeruginosa*, however, other work showed that a non-selective growth medium could also be used instead due to effectiveness of the disinfection procedure. In all this work three replicates were prepared for each inoculum at each timepoint.

2.3 Other method

All UV-vis absorbance measurements were made using an Agilent Technologies CARY 60 UV–vis spectrophotometer (Agilent, Stockport, UK), and all digital photography was taken using a Canon 7D digital camera (Canon Inc., London, UK) lit with a D65 daylight lamp.[20]

S3 Characterisation of the 3D printed XB indicator

XB indicator images in deprotonated and protonated forms

The XB CO_2 indicator was photographed in the absence of CO_2 (in Argon) and in its present (100% CO_2) and the results of this work are illustrated in Figure S3.1 below.

Figure S3.1. From left to right photographic images of the 12 mm square 3D printed, XB CO_2 indicator in the absence of CO_2 (in Argon) and in its present (100% CO_2), respectively.

Response and recovery times

Response and recovery times of the 3D printed XB indicator film was carried out using the CARY 60 UV–vis spectrophotometer. Absorbance of the indicator film was monitored at 620 nm, which is the λ_{max} of the deprotonated (D^-) blue form of the XB indicator. 3D printed XB indicator film was placed in a gas cell and alternative gas stream of air and 100% CO_2 was purged through the gas cell while monitoring the absorbance.

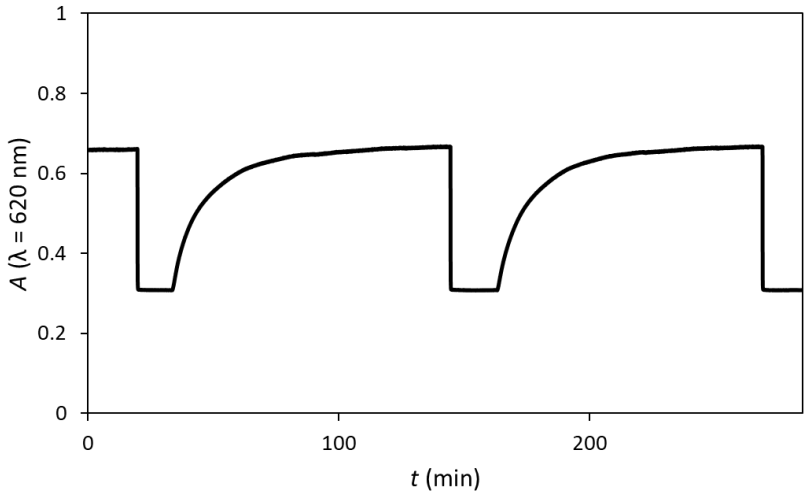

Figure S3.2. Response and recovery spectra of the 3D printed XB indicator film recorded under an alternative gas stream of Air and 100% CO_2, revealing a 50% response time of $t_{50\downarrow}$ = 0.17 min and 50% recovery time of $t_{50\uparrow}$ = 8.3 min.

CO_2 sensitivity

The sensitivity of the 3D printed XB indicator film was measured by monitoring its colour, using a Canon 7D digital camera, as a function of $\%CO_2$ in the ambient atmosphere, all carried out at 30°C. Thus, under these conditions the 3D printed XB indicator film was placed in a gas cell and exposed to a range of different CO_2 levels, varying from 0 to 100% CO_2. The photographic images taken of the XB indicator in an atmosphere containing 0, 0.04, 0.1, 1, 5, and 100% CO_2, respectively are illustrated in Figure S3.3(a).

Recent work[21] has shown that, for simple colour-changing systems such as for the XB indicator, digital photography coupled with digital colour analysis (DCA) can be used to generate apparent absorbance *A'* value which is directly proportional to their absorbance *A*. Based on the red (R), green (G) and blue (B), i.e., RGB, components of the image, *A'* can be calculated using eqn (S1) below:

$$A' = \log\{255/RGB\} \quad \text{(S1)}$$

For a blue colour dye, as used here, the red RGB was used to calculate the associated value of *A'* for the XB indicator. *R* vs % CO_2 plot shown in Figure S3.3(b) was generated by using the *A'* calculated from the Figure S3.3(a) and the following the equation which has been shown for many CO_2 indicators to relate the colour of the indicator, as measured by its value of *A'* to the ambient $\%CO_2$.[21-24]

$$R = (A'_0 - A')/(A' - A'_\infty) = \alpha\ \%\ CO_2 \quad \text{(S2)}$$

where, here, A'_0 is the value of the apparent absorbance of the 3D printed XB indicator at $\%\ CO_2$=0 (i.e., when all the dye is in its deprotonated, blue form), and A'_∞ is the apparent absorbance of the film when all of dye is in its protonated (yellow) form, i.e., when $\%CO_2 = \infty$. The latter apparent absorbance is assumed to be that measured when the $\%\ CO_2$= 100% and the film is bright yellow coloured.

When the concentrations of the protonated and deprotonated forms of the dye are the same, i.e., when *R* = 1, the 3D printed XB indicator will be halfway through its blue to yellow colour change, i.e., green. The value of the $\%CO_2$ when this occurs is usually referred to $\%CO_2$ (S=1/2), where, from eqn (S2), $\%CO_2$ (S=1/2) = 1/α. For the XB indicator the value of $\alpha = 0.79\ \%^{-1}$ and so the value of $\%CO_2$ (S=1/2) is = 1.3 %.

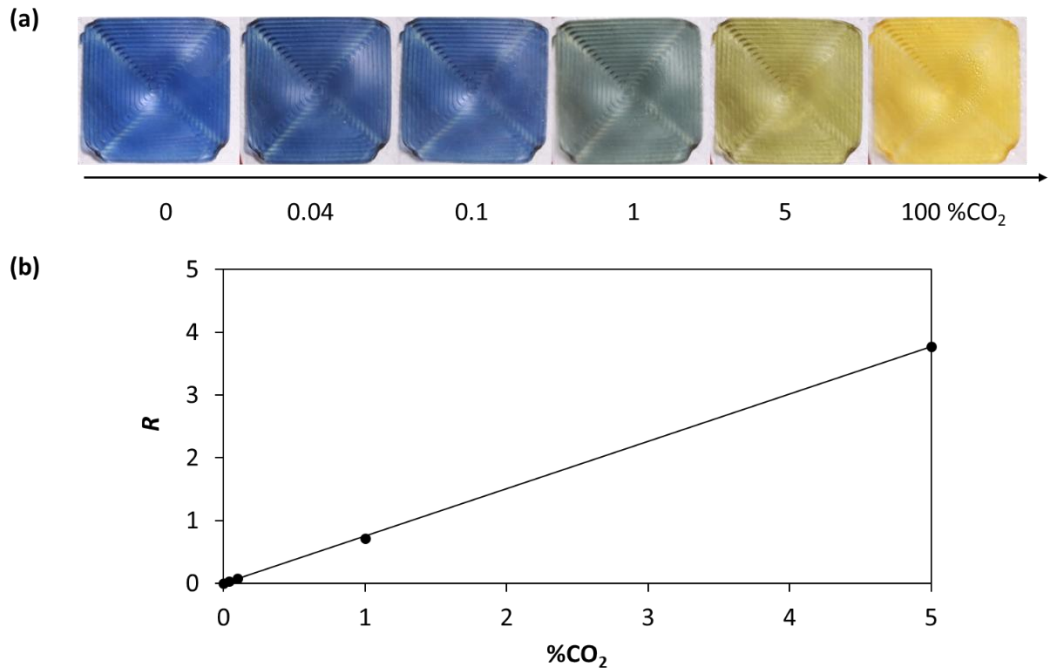

Figure S3.3(a). Digital photographs of the 3D printed XB indicator film in a 30°C gas cell as a function of the $\%CO_2$ in the 100% humid gas stream. **(b).** Plot of *R* vs $\%CO_2$, where *R* values were calculated using eqn (S2) and apparent absorbance values, *A'* derived from colour analysis of the images given in Figure S3.3(a). The *R* vs $\%CO_2$ plot yields a gradient of 0.79 ± 0.05 $\%^{-1}$ (R^2 = 0.9962).

S4 Biomass of Biofilm as a function of incubation time

The formation of a biofilm on the wounded porcine skin was confirmed by inoculating different samples with 20 μl of 10^6 CFU ml^{-1} of *P. aeruginosa*, and then incubating them for the following different times, 0, 6, 12, 24, 30 and 48 h, after which the biomass of the biofilm colony was determined. Thus, after incubation, each sample was immersed in 1 ml of phosphate buffer solution, PBS, to wash off the planktonic bacteria and then soaked in 1 ml of 0.1 wt% crystal violet, CV, solution for 20 min, before rinsing with 1 ml PBS twice to wash off any excess dye. Each stained sample was then thoroughly vortexed in 1 mL of 95% ethanol, to extract out the CV which had stained the biofilm and then removed from the solution. The CV containing ethanolic solution was then diluted 1 in10 times to allow its optical density, OD, i.e. absorbance, to be measured at 570 nm in a 96-well microliter plate using a microplate reader. As with all this work 3 repeats were performed for each sample and the subsequent plot of the results in the form of the OD at 570 nm, i.e. OD_{570}, vs incubation time, *t*, is illustrated in Figure S4.1. These results, and those generated using non-inoculated wounded porcine skin samples, show that the CV stained the wounded porcine skin samples to some extent even before biofilm formation, thereby producing a background OD_{570} value of ca. 0.3. However, with incubation time the value of OD_{570}, which is usually taken as a direct measure of the biomass of the colony biofilm present,[7] gradually increased with incubation time after an initial period of ca. 12 h. These results are consistent with those reported by others using the same microbial species but grown on a colony biofilm model, namely a slow by steady increase in biomass with incubation time.[7] The results in Figure S4.1 show that biofilm formation begins after ca. 12 h, which is after the time the CO_2 indicator exhibits an apparent absorbance of ca. 0.6 and the microbial burden reaches a value of ca. 10^6 g^{-1}. These findings appear consistent with the wound infection continuum illustrated in Figure 1, namely biofilm formation is closely associated with the microbial load exceeding the critical colonisation threshold (CCT) and infection taking hold and that the CO_2 indicator is able to act as an early signal of the rapid microbial colonization of the wound that leads to biofilm formation and infection.[25, 26]

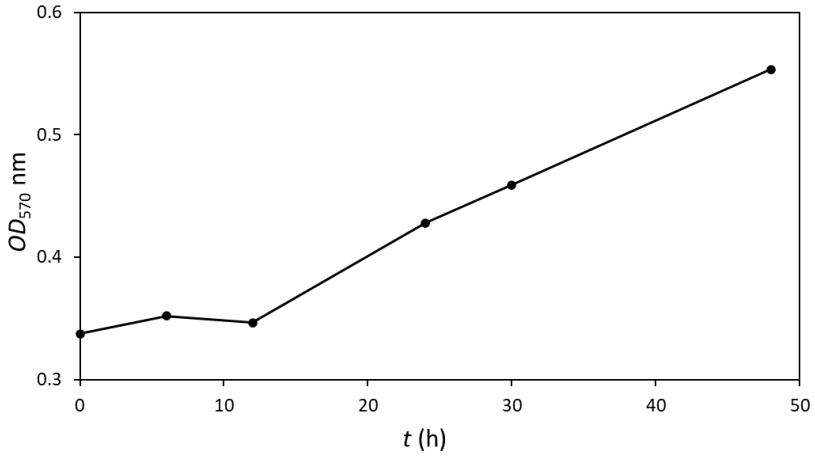

Figure S4.1. Measured variation in the optical density due to CV, i.e. OD_{570}, that has stained the porcine skin sample and any biofilm, as a function of incubation time, *t*.

S5 Different microbial pathogens

Most wounds are colonized by multiple microbial species, but at sufficiently low levels they do not inhibit the healing process, i.e. well below the CCT.[7] It is important therefore to demonstrate that the CO_2 indicator works with wound infection microbial species other than *P. aeruginosa*. Thus, in another set of experiments using the same system as illustrated in Figure S2.2, the porcine skin wound was inoculated with 20 μl of 10^5 CFU ml^{-1} of each of the following wound pathogenic species, *E. faecium*, *A. baumannii, S. pyogenes, C. albicans and S. aureus*, with *P. aeruginosa* for comparison purposes. The resulting photographs of the CO_2 indicator as a function of incubation time are illustrated in Figure S5.1, from which the plots of apparent absorbance of the indicator film, i.e. *A'*, as a function incubation time, *t*, illustrated in Figure S5.2 were generated.

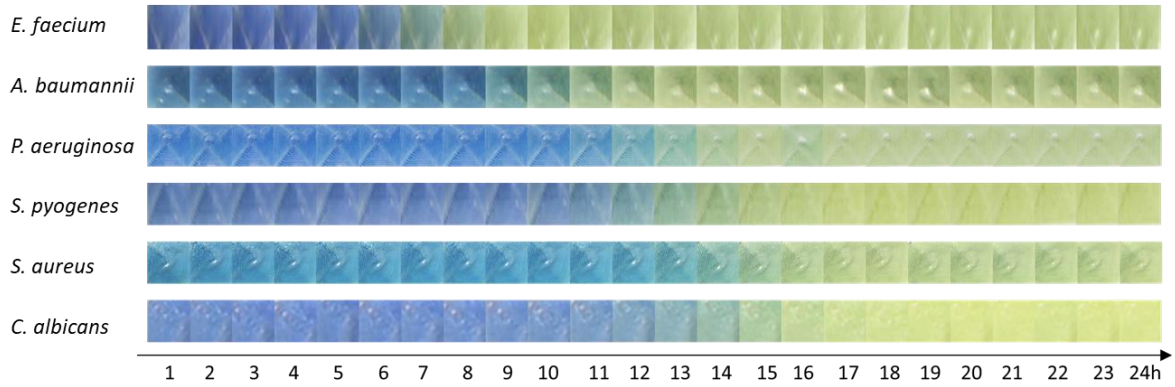

Figure S5.1. Photographic images of the 3D printed XB CO_2 indicator in the dressed, inoculated wound illustrated in Figure S2.2 recorded as a function of incubation time, *t*, at 30 °C, *t*, every hour for 24 h, with an initial inoculum of 20 μl of 10^5 CFU ml^{-1} of each of the following wound pathogenic species (from top to bottom), *E. faecium, A. baumannii, P. aeruginosa, S. pyogenes, S. aureus, and C. albicans,* respectively.

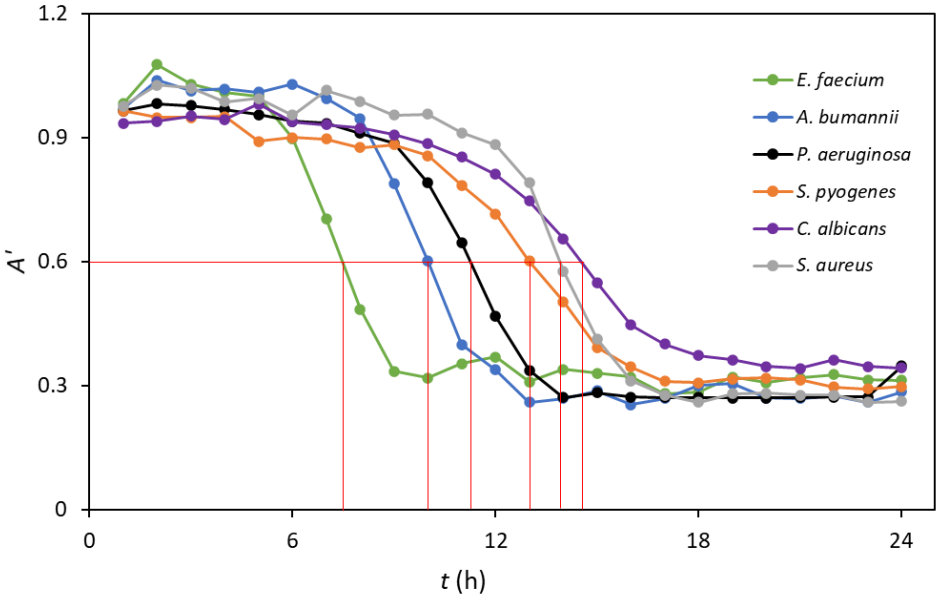

Figure S5.2. Plots of apparent absorbance of the indicator film, i.e. *A'*, calculated using eqn (S1), as a function incubation time, *t*, for dressed wounded, porcine skin inoculated with 20 μl loaded 10^5 CFU ml^{-1} of (from left to right): *E. faecium, A. baumannii, P. aeruginosa, S. pyogenes, S. aureus, and C. albicans,* respectively. These plots were generated from the digital images illustrated in Figure S5.1.

In an otherwise identical set of experiments, as described for Figure S5.1, the microbial load (CFU g^{-1}) of each different pathogen was determined for the following incubation times, 0, 6, 12, 24 and 30 h. The subsequent plots of log (CFU g^{-1}) of microbial pathogen vs incubation time are illustrated in Figure S5.3. From the data in Figures S5.2 and S5.3 it is possible to determine the values of t(A' = 0.6) and $t(10^6)$, respectively foreach different microbial species. The subsequent plot of t(A' = 0.6) vs $t(10^6)$ yielded a good straight line with zero intercept as illustrated in Figure S5.4 below.

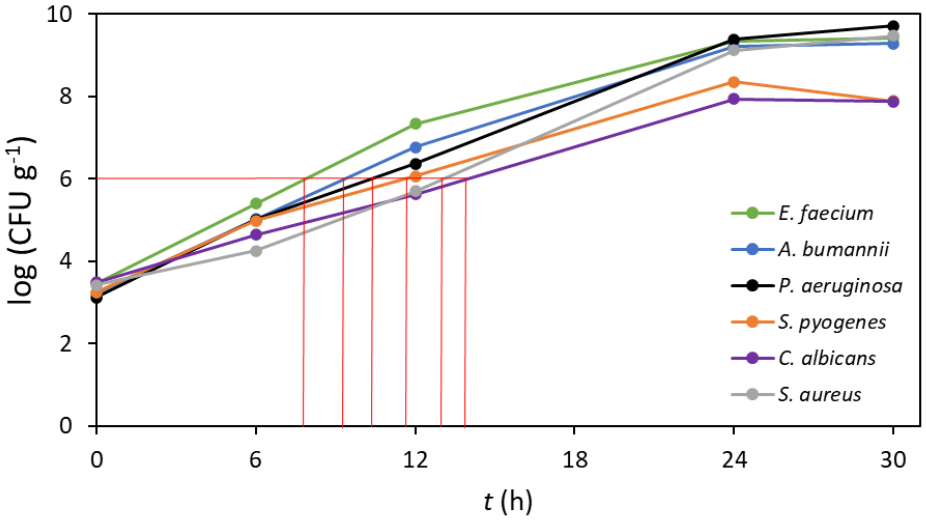

Figure S5.3. Plot of measured log(CFU g^{-1}) of (from top to bottom): *E. faecium, A. baumannii, P. aeruginosa, S. pyogenes, S. aureus, and C. albicans,* respectively, as a function of incubation time, *t,* for an identical set of dressed, inoculated porcine skin wound samples as used to generate the data in Figure S5.1; thus, with samples incubated at 30 °C, with an initial inoculum 10^5 CFU ml^{-1}, respectively.

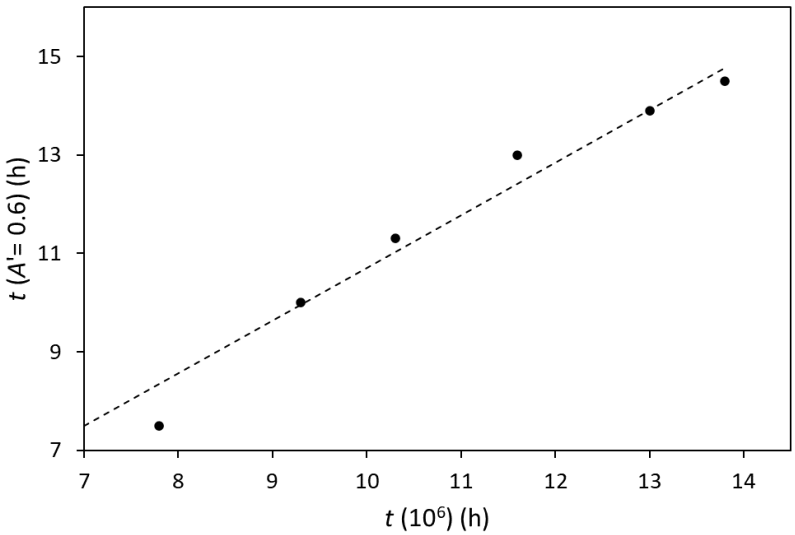

Figure S5.4. Plot of t(A' = 0.6) – from Figure S5.2 - vs. $t(10^6)$ – from Figure S5.3.

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