Development of a flow system for studying biofilm formation on medical devices with microcalorimetry

Jawal Said[a,*], Michael Walker[b], David Parsons[b], Paul Stapleton[a], Anthony E. Beezer[a], Simon Gaisford[a*]

[a]UCL School of Pharmacy, University College London, 29-39 Brunswick Square, London, WC1N 1AX, UK

[b]ConvaTec GDC, First Avenue, Deeside Industrial Park, Deeside, Flintshire, CH5 2NU, UK

*Corresponding authors

email: s.gaisford@ucl.ac.uk, jawal.said.11@ucl.ac.uk

Tel: +44(0)20 7753 5863

Fax: +44(0)20 7753 5942

Abstract

Isothermal microcalorimetry (IMC) is particularly suited to the study of microbiological samples in complex or heterogeneous environments because it does not require optical clarity of the sample and can detect metabolic activity from as few as 10^4 CFU/mL cells. While the use of IMC for studying planktonic cultures is well established, in the clinical environment bacteria are most likely to be present as biofilms. Biofilm prevention and eradication present a number of challenges to designers and users of medical devices and implants, since bacteria in biofilm colonies are usually more resistant to antimicrobial agents. Analytical tools that facilitate investigation of biofilm formation are therefore extremely useful. While it is possible to study pre-prepared biofilms in closed ampoules, better correlation with in-vivo behaviour can be achieved using a system in which the bacterial suspension is flowing. Here, we discuss the potential of flow calorimetry for studying biofilms and report the development of a simple flow system that can be housed in a microcalorimeter. The use of the flow system is demonstrated with biofilms of *Staphylococcus aureus.*

Key words

1. Introduction

Isothermal microcalorimetry (IMC) is a technique that permits accurate measurement of extremely small powers (<1 μW) and which imposes no requirements on the physical nature of the sample under investigation (the sample can be solid, liquid or inhomogeneous for instance). These qualities make it ideally suited to the study of bacterial growth and metabolism in real-time. In particular, the exquisite sensitivity means as few as 10^4 metabolically active cells can be detected [1]. Further, it is possible to monitor bacterial activity in the presence of antimicrobial agents and/or medical devices and implants because there is no requirement for optical clarity of the test system.

IMC has been used to follow microbiological activity in a wide range of applications, including for monitoring and quantifying soil microbial activity and contamination [2-3], treatment of sewage [4], detecting infection in and contamination of clinical products and samples [5], quantifying the efficacy of antimicrobial compounds [6-7], including the mode of action of bacteriostatic or bactericidal compounds [8] and antiviral compounds [9-10] and looking at spoilage of food [11]. A few studies have been conducted on defined mixed cultures with the aim of investigating the relationship between two or more bacteria (for instance, [12-14]).

An area of particular clinical interest is in monitoring attachment and colonisation of bacteria to form biofilms on the surface of medical implants and devices. Indwelling medical devices are crucial components of modern healthcare practice. For example, at any one time up to 10% of patients in a hospital may have an indwelling catheter [15]. However, these devices create a pathway into the warm, nutrient rich environment inside a patient that is usually inaccessible to bacteria, thus acting as a conduit between the patient and the non-sterile outside environment [16-17]. Microorganisms may also irreversibly adhere to the surfaces of medical devices, forming biofilms [18]. Biofilms are sessile communities of microorganisms typically held together by an extracellular matrix [19]. Studies using *Pseudomonas aeruginosa* have shown that biofilm development proceeds in stages including initial attachment, production of extrapolymeric substances (EPS), early development of biofilm architecture, maturation of biofilm architecture and finally release of cells from the mature biofilm [20-21]. The presence of biofilms in medical devices poses a threat to patients as a result of the inherent resistance to antimicrobials [22]. Biofilms may also act as a starting point for further infection as cells are released or sloughed off the biofilm [23]. Urinary tract infections are the most common hospital acquired infections [24] and the length of time that catheters are used influences the likelihood of infection. For example catheterisation for more than four weeks results in urinary tract infections in almost all cases [15]. However,

even brief catheterisation, of less than ten days, resulted in 26% of patients contracting urinary tract infections [25].

1.1. In vitro medical device testing

Since in vivo determination of biofilm formation and activity is difficult, analytical methods are required to understand in vitro how biofilms form on medical devices and to determine how effective antibiofilm technologies or eradication measures may be [17]. Biofilms can be difficult to generate in vitro, because the drivers that direct bacteria to form biofilms are not always clear, but environmental stresses, including shear forces, are known to play a role. Biofilms are usually created in microbial bioreactors, typically of one of three designs; closed batch systems with limited substrate, continuous flow stirred tank reactors (CFSTR) and plug flow reactors (PFR) [26]. With batch systems, the substrate concentration declines while there is a concomitant increase in biomass. With a CFSTR system, the quantities of substrate and biological products remain constant because a continuous flow of fresh substrate is introduced at the same rate as accumulating metabolic by-products are removed [26]. A PFR system is 'fed' initially and there is continuous flow to a waste vessel but conditions are otherwise similar to batch systems with accumulated biomass and reduced substrate over the course of experiments [26].

Irrespective of their basic design, flow models typically rely on shear forces to drive biofilm formation and utilise plastic inserts (or coupons) that act as a surface to initiate and support biofilm growth. The coupons are removable, which allows analysis of the biofilm with conventional microbiological methods such as microscopy (including scanning and transmission electron microscopy or confocal laser scanning microscopy), staining or viable colony counting. One drawback of this approach is that they all require removal of the biofilm from its natural environment prior to analysis, which may cause changes in structure and/or viability. It is also not possible to monitor the processes of biofilm growth and establishment in real time.

Direct, non-invasive methods of monitoring biofilms would, in principle, allow insight into the entire process of biofilm development in real-time without interference. Methods have been developed based on differential turbidity, heat transfer, bioluminescence, computerized image analysis and spectroscopy [27]. However, the challenge lies in correlating changes in measured parameters with actual processes in the biofilm. Flemming [28] categorises techniques used to monitor biofilms as follows; (i) systems that detect biofilm by deposition of material and changes in thickness of layer without differentiating between biotic and abiotic components (ii) systems that can distinguish between biotic and abiotic components

and (iii) systems that provide detailed information about the chemical composition of the microorganisms involved. Janknecht and Melo [27] add a fourth category; that of systems that monitor metabolic activity. IMC falls within the latter category (and may also be considered as part of the second category as abiotic components would not register any metabolic activity).

1.2. Isothermal microcalorimetry for biofilm studies

Several recent studies report the use of IMC for monitoring biofilm activity suggesting the technique has much potential [29]. For instance, Clauss et al [30] monitored biofilms of *Staphylococcus aureus* growing on human and bovine bone grafts, while Astasov-Frauenhoffer et al [31] investigated variability and dynamics of a triple-species biofilm and determined efficacy of amoxicillin and metronidazole combinations against biofilms of various species [32]. Said et al [33] determined the efficacy and mechanism of action of an anti-biofilm wound dressing. Lerchner et al [34] note, however, that despite the widespread use of IMC for studying planktonic cells, its use for biofilm investigation is rarely considered. This may be because the data are complex to interpret, but may also be ascribed to experimental limitations. IMC experiments are typically conducted in closed ampoules, and this arrangement is not particularly suited to the study of biofilms as the limited nutrient supply may result in the bacteria entering a stationary phase and/or that metabolic by-products poison the medium, leading to cell death. It is also necessary to generate the biofilm on a coupon as described above prior to calorimetric measurement, so the processes of formation and growth are not observed as they occur prior to experimental measurement.

1.3. Flow calorimetry for biofilm studies

A more effective arrangement would allow fresh medium and/or bacterial culture to be circulated from an external reservoir through the calorimetric chamber, keeping nutrient levels high. Such systems are known as flow calorimeters, and fall under the category of plug flow reactors as defined above. They can be configured to circulate material back to the reservoir (continuous-loop) or to flow material to waste. Flow calorimeters have many advantages that allow biofilms to form and to remain viable over extended time periods; nutrient levels and/or compositions can be optimised, planktonic cells can be continuously added, shear forces may exist in the circulating liquid and it is possible to maintain or control pH, preventing conditions arising that limit cell growth. From the perspective of interrogating biofilms, it is possible to add agents that modify metabolic rates or pathways or which are designed to eradicate biofilms. Antimicrobial agents may also be titrated into the bioreactor, and so it is easy to get a direct measurement of antimicrobial efficacy, including determination of minimum inhibitory and minimum bactericidal concentrations.

Despite these advantages, flow calorimetry has not been widely applied to biofilm investigation. Lerchner et al [34] discuss a flow calorimeter based on a solid-state (or chip) design and use it to study *Pseudomonas putida* biofilms, although in this case the biofilms were cultured prior to calorimetric measurement. Mariana et al [35] used the same chip calorimeter to monitor eradication of *P. putida* biofilms with antibiotics. Morais et al [36] have also used chip calorimeters to investigate antimicrobial efficacy of biofilms. Peitzsch et al [37] used flow calorimetry to study biofilms of *Escherichia coli*. Lerchner et al [34] discuss some of the potential limitations hindering widespread use of flow calorimetry; the cost of the calorimeter can be high, they are not suited to high throughput studies, experiments may take days to weeks and it can be difficult to manipulate and investigate substratum effects on the biofilm. Additionally, it can be difficult to maintain anaerobic conditions and there is a need to minimise the temperature difference between the external reservoir and the calorimeter.

Since many commercially available IMC instruments are designed to accept removable ampoules, it is possible to consider the development of a system that flows liquid through the ampoule from an external reservoir. The ampoule is housed in the calorimeter and so power data can be recorded from the medium flowing through it. Constructing a flow system in this way involves relatively low cost (assuming the calorimeter itself is already available). Here, we outline the design simple flow system developed to fit into a commercial isothermal calorimeter. It is constructed so that different materials can be connected to the flow lines within the ampoule itself. In this way, the system may be used to investigate biofilm formation on a wide range of materials used for medical tubing. It would be equally be feasible to load the ampoule with pelletised samples so test biofilm formation on any material used for fabricating medical devices and implants. We demonstrate application of the flow system to biofilm formation on the internal walls of silicone tubing.

2. Material and methods

All experiments were conducted in a 2277 Thermal Activity Monitor (TAM, TA Instruments LLC). The instrument utilises removable ampoules, which means it is relatively easy to design and construct specific apparatus to fit in the measuring position. In this case, the flow system was constructed to fit into the 20 mL calorimeter.

2.1. Development of the flow reactor

The objective of the work was to create a plug flow system to facilitate formation and maintenance of a biofilm within medical tubing. The simple system designed (shown

schematically in Figure 1) comprised an external bioreactor, the contents of which were circulated through an ampoule housed within the TAM. The bioreactor was maintained at 37 ^{o}C by a jacketed water bath. Silicone tubing (Tygon® peristaltic pump tubing, I.D. 2.06 mm) was used to attach the bioreactor to the ampoule. The ampoule itself was machined from stainless steel and was of 20 mL volume. Two stainless steel tubes were set through the lid of the ampoule; in use, the tubing being tested (up to 15 cm in length) can be connected to the inlet and outlet lines and coiled within the ampoule. The ampoule was filled with sterile distilled water (10 mL) to act as a heat-transfer fluid enabling heat exchange with the calorimeter. This design maximises versatility of the system because tubing of different material can be connected or it is possible simply to fill the ampoule with pellets of a sample. A heat exchange coil was incorporated before the inlet tube, and mounted inside the body of the calorimeter, to ensure that medium arriving from the bioreactor was at exactly the same temperature as the calorimeter. A peristaltic pump (Gilson Minipuls 3) was mounted in the outflow line and used to circulate media at 4 mL/h (flow rates suggested not to cause noise in the baseline signal of flow calorimeters range from 3.5 mL/h [38] to 100 mL/h [39], so the lower end of this range was selected).

All experiments were performed at 37 ^{o}C. Data were captured with the dedicated software package Digitam 4.1 (1 data point every 10 s, amplifier setting 300 μW). The instrument was calibrated periodically by the electrical substitution method (wherein a known current is passed through a calibrated resistor mounted at the base of the calorimeter). Data were analysed with Origin 8.1 (Microcal Software Inc).

2.2. Biofilm models

The organism used was *Staphylococcus aureus* NCIMB 9518. The bacterial culture, consisting of tryptone soya broth (TSB, 200 mL, Thermofisher) inoculated with *S. aureus* to a final population density of 1×10^6 CFU/mL, was maintained in the bioreactor with constant stirring and circulated through the calorimetric ampoule at 4 mL/h. Anaerobic conditions were maintained by placing a layer of mineral oil on the surface of the contents of the bioreactor to minimise rates of gas exchange with the environment. The power signal from the calorimeter was used as an indicator of biofilm formation during the experiment. After each experiment the catheter tubing was removed and several tests were performed to visualise and confirm biofilm formation.

Staining: Crystal violet staining was used to visualise biofilm presence in the tubing following calorimetric experimentation [40]. Crystal violet solution (100 μL, Fisher) was pipetted through the tubing. The stained section was then thoroughly rinsed with distilled water until

the water running off was visibly clear. The same procedure was applied to an equivalent length of clean tubing in order to compare the extent of 'background' staining.

Colony counting: The presence of viable bacteria in the biofilms was confirmed by viable colony counting following the method outlined by Nowatzki et al [24]. The outer surface of the catheter section was wiped with 70% ethanol then lengths (2 cm) were cut and rinsed gently with phosphate buffered saline (PBS, Thermofisher) to remove any non-adherent cells. The pieces were then transferred to bijous containing PBS (5 mL). The samples were subjected to three cycles of vortexing for one minute followed by sonication for five minutes. Colony counts were determined by serially diluting the PBS from the ampoules and spread plating onto iso-sensitest agar (Thermofisher). Colonies were counted following 16h incubation at 37 ^{o}C. Counts are expressed as CFU/cm^2 of inner tubing surface.

Imaging: Scanning electron microscopy (SEM) was used to image biofilm formation on silicone tubing walls. Lengths of tubing (2 cm) were cut and gently rinsed in saline to remove planktonic cells. The tubing was then placed into 2.5% glutaraldehyde solution overnight before soaking in successive concentrations of ethanol (10%, 25%, 50%, 70%, 90% and 100%). Tubing was kept in each concentration of ethanol for 10 minutes. Samples of tubing were then dehydrated in alcohol and air dried before being adhered onto an SEM stub with carbon discs (both Agar Scientific, UK). Prepared samples were then coated with gold (10nm) in a Quorum Q150T Sputter coater (Quorum Technologies, UK), then transferred to the FEI Quanta 200F (Eindhoven, The Netherlands) SEM for imaging.

Antimicrobial challenge: Further confirmation of biofilm presence and activity during calorimetric measurement was achieved by challenge with silver nitrate (AgNO$_3$) solution [34, 40]. Biofilms that had been established in the tubing in the calorimeter were thoroughly rinsed in situ by flowing sterile distilled water (200 mL) at the fastest rate achievable by the pump (2 mL/min). The external bioreactor was then removed, cleaned and autoclaved before being charged with fresh TSB/AgNO$_3$ solution (1x10^{-4}M). The AgNO3 solution was circulated over the biofilm at 4 mL/h.

3. Results and discussion

The first aim in developing the flow microcalorimeter system was to achieve a stable baseline power signal when sterile medium was being circulated, and this was achieved with a flow rate of 4 mL/h (Figure 2). There is some fluctuation in the signal, most probably due to small changes in room temperature during the measurement period. These fluctuations were minimised by incorporating the heat-exchange coil in the inlet line of the system. A

significant advantage of the design is that it can be removed from the calorimeter itself, allowing sterilisation of the ampoule.

Loading the bioreactor with *S. aureus* culture resulted in an exothermic peak after a lag period of ca. 5h, which increased to reach a plateau after ca. 12 h, Figure 2. It has been established that growth of planktonic *S. aureus* cells in a closed ampoule results in an exothermic power that rapidly reduces to a zero power once nutrients in the medium are exhausted and/or the metabolites produced by the bacteria have poisoned the medium [41]. The data recorded here reach a plateau, meaning the system is undergoing a zero-order kinetic process; in other words, there must be on-going metabolic activity within the calorimetric ampoule, from biofilm activity. Bacteria existing in a biofilm colony are not exponentially increasing in number and are shielded from toxic metabolites but since fresh medium is constantly being circulated over the biofilm the bacteria have a nutrient source to sustain metabolic activity.

To confirm that the power output was from sessile, surface-attached cells and not because of other factors, such as increased friction due to biomass or simply the activity of planktonic cells, the silicone tubing was rinsed extensively by flowing 200 mL of sterile distilled water at the maximum flow rate achievable with the peristaltic pump. Fresh TSB, dosed with 1×10^{-4} M silver nitrate solution was then introduced into the bioreactor and circulated through the calorimeter at 4 mL/h. Said et al [33] have shown previously that silver nitrate solution at this concentration is bactericidal against planktonic *S. aureus*, but has no bactericidal effect against an established biofilm. The resulting power data are shown in Figure 3. It is apparent that after a small initial disturbance, the power output rapidly returned to that of the steady state achieved initially and that the silver nitrate exerted no effect, supporting the hypothesis that a biofilm had been established with constant metabolic activity.

In a final calorimetric experiment, TSB was replaced with sterile PBS and circulated through the established biofilm. Since PBS contains nothing of nutritional value to the cells, metabolic activity quickly reduced to zero, Figure 4.

Following calorimetric experimentation, the silicone tubing was removed from the ampoule and analysed. Staining with crystal violet showed clear evidence of cell attachment to the tubing, Figure 5. SEM images of the tubing showed clear attachment of cells to the inner walls, Figure 6. Viable cell counts from of a section (2 cm) of tubing were 3×10^9 CFU/cm^2, which corresponds to approximately 3×10^{10} CFU for the total length of tubing, assuming uniform cell coverage. The inner volume of the catheter was approximately 1 mL, which

implies a cell density of 3×10^{10} CFU/mL. If these cells were in a highly metabolically active planktonic state a much higher power would be expected, further supporting the notion that a biofilm had formed.

4. Summary

Isothermal microcalorimetry has much potential for the study of biofilms, but its use is frequently restricted to monitoring activity of established biofilms because it is difficult to grow and maintain biofilms in closed ampoules. Use of a flow system offers the opportunity to provide a continuous supply of nutrients from an external reservoir and so to conduct experiments over longer time periods. This permits formation of the biofilm within the ampoule and so monitoring of the power changes directly, in real time. The flow system reported here is versatile, so easily adapted to study a range of materials, and removable, so easy to sterilise. Flowing an *S. aureus* culture through the system resulted in an increasing exothermic power, reaching a plateau after ca. 12 h. It was established that the bacteria were in biofilm colony at this stage and that they showed increased resistance to antibacterial agents. The flow system can thus be used to establish and monitor biofilm formation and growth on a range of medical-grade plastics.

5. Funding

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Figure 1. Schematic representation of the flow calorimeter

Figure 2. Power-time data for the control (TSB) and for a suspension of *S. aureus*

Figure 3. Power-time data for showing formation of the biofilm (black) and the response of an established biofilm to exposure to AgNO$_3$ (1 x 10^{-4} M, Grey)

Figure 4. Power-time data showing the response of an established biofilm to a nutrient-poor medium (PBS)

Figure 5. Sections of silicone tubing after staining with crystal violet solution. Left; tubing exposed to S. aureus suspension. Right; Control tubing exposed to TSB only

Figure 6. SEM image of a cross-section of tubing exposed to S. aureus suspension, showing clear evidence of biofilm formation.
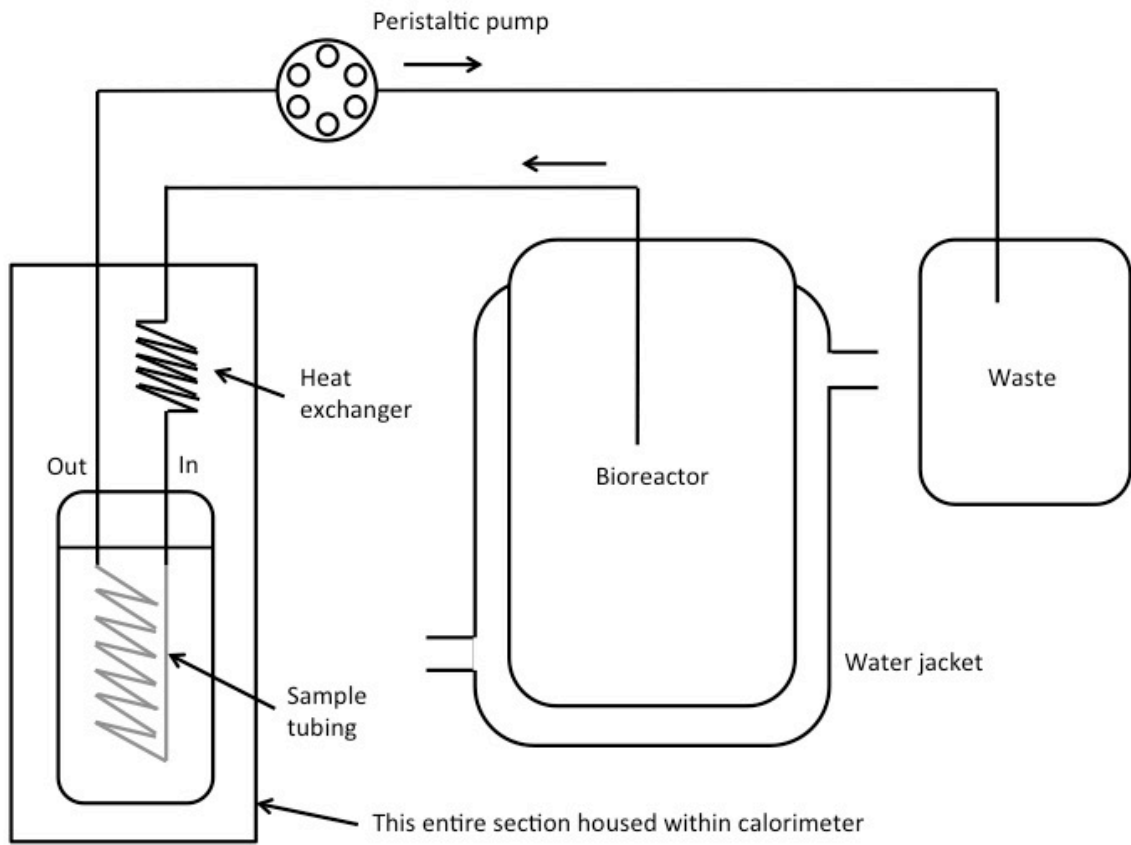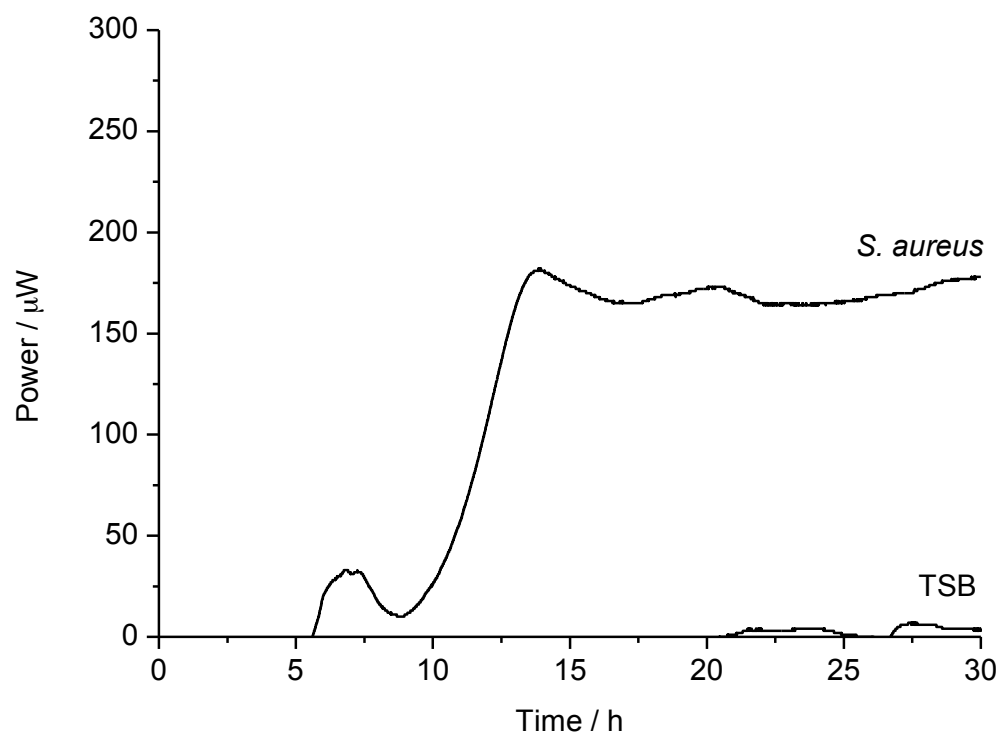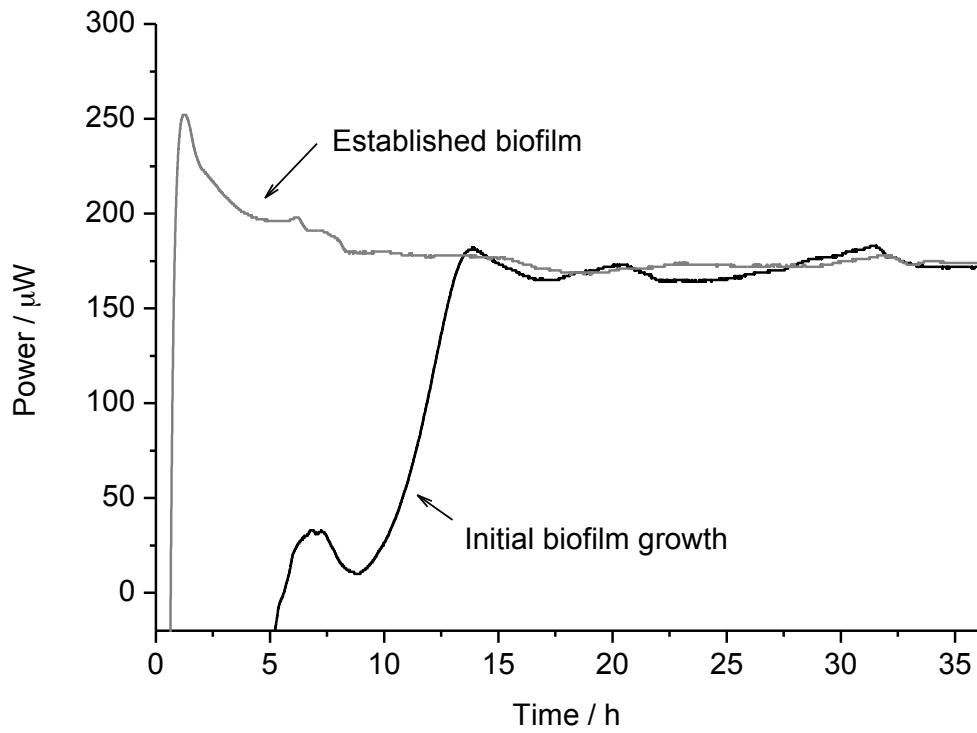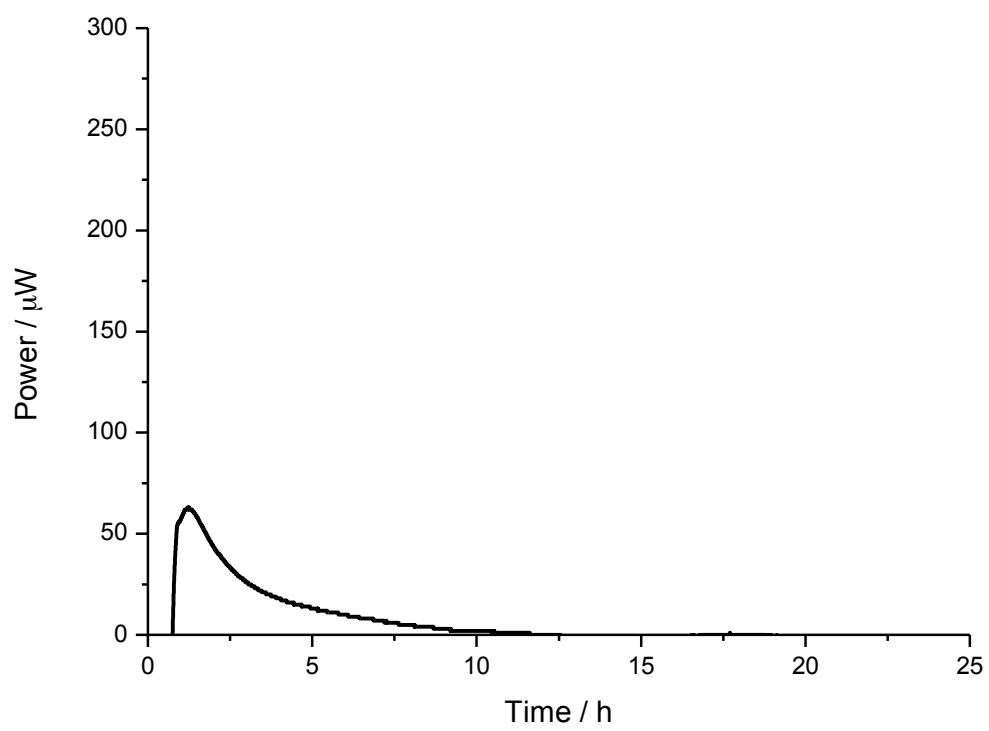
Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

HV	spot	det	WD	mag □	HFW	pressure	⟶ 2 mm ⟶	
10.00 kV	2.0	ETD	11.9 mm	34 x	7.63 mm	2.12e-3 Pa	RT	Ref:473.3

Figure 6.