Protein-conjugated microbubbles for the selective targeting of
*S. aureus* biofilms

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ABSTRACT

*Staphylococcus aureus* (*S. aureus*) is an important human pathogen and a common cause of bloodstream infection. The ability of *S. aureus* to form biofilms, particularly on medical devices, makes treatment difficult, as does its tendency to spread within the body and cause secondary foci of infection. Prolonged courses of intravenous antimicrobial treatment are usually required for serious *S. aureus* infections. This work investigates the in vitro attachment of microbubbles to *S. aureus* biofilms via a novel Affimer protein, ACIfA1, which targets the clumping factor A (ClfA) virulence factor—a cell-wall anchored protein associated with surface attachment. Microbubbles (MBs) are micron-sized gas-filled bubbles encapsulated by a lipid, polymer, or protein monolayer or other surfactant-based material. Affimers are small (~12 kDa) heat-stable binding proteins developed as replacements for antibodies. The binding kinetics of ACIfA1 against *S. aureus* ClfA showed strong binding affinity (*Kd = 62 ± 3 nM*). ACIfA1 was then shown to bind *S. aureus* biofilms under flow conditions both as a free ligand and when bound to microparticles (polymer beads or microbubbles). Microbubbles functionalized with ACIfA1 demonstrated an 8-fold increase in binding compared to microparticles functionalized with an identical Affimer scaffold but lacking the recognition groups. Bound MBs were able to withstand flow rates of 250 μL/min. Finally, ultrasound was applied to burst the biofilm bound MBs to determine whether this would lead to biofilm biomass loss or cell death. Application of a 2.25 MHz ultrasound profile (with a peak negative pressure of 0.8 MPa and consisting of a 22-cycle sine wave, at a pulse repetition rate of 10 kHz) for 2 s to a biofilm decorated with targeted MBs, led to a 25% increase in biomass loss and a concomitant 8% increase in dead cell count. The results of this work show that Affimers can be developed to target *S. aureus* biofilms and that such Affimers can be attached to contrast agents such as microbubbles or polymer beads and offer potential, with some optimization, for drug-free biofilm treatment.

1. Introduction

*Staphylococcus aureus* (*S. aureus*) is a common pathogen, frequently associated with the formation of biofilms in intravascular catheters or organs within the body. It is one of the most serious causes of bloodstream infection with mortality rates of 17–45.7%, and causes approximately 12,700 and 119,000 cases per year in England and the United States of America, respectively [1–5]. Bacteria are known to exist in three common states; planktonic, non-surface attached aggregates, and...
Microbubbles (MBs) are micron-sized gas-filled bubbles encapsulated by a lipid monolayer or other surfactant-based material [14,15]. MBs have been engineered to be used as ultrasound contrast agents (UCAs) and are in routine clinical use, for example during the echocardiographic examination of cardiac blood flow. Clinically approved MBs such as Definity and SonoVue have mean diameters between 1 and 3 μm allowing flow through the vasculature [16–20].

Microbubbles have been targeted against tumour vasculature using BR55, VEGFR1/2, CD-31, PD-L1, FSHR, αvβ3 integrin targets, and other relevant targets [21–33]. Most studies have focused on using targeting to aid the identification of tumors through enhanced ultrasound imaging, with improved therapeutic delivery of chemotherapeutic agents also demonstrated [26]. Targeted MBs have also been utilized as a non-invasive method of assessing inflammation sites by targeting activated leukocytes [34–36]. Finally, alternative strategies for achieving microbubble localization have been developed which involve the incorporation of magnetic nanoparticles within the MB shell [37–39]. The use of an external magnet causes the accumulation of MBs at the desired site.

Targeting of MBs in the context of human infection has seen comparatively little research interest within the same time frame and only two such examples have been identified. Matter et al. demonstrated the synergy of MBs and targeted monoclonal antibodies (mAbs) as a novel method of non-invasive high-frequency ultrasound imaging of biofilms for structural investigations [40]. The second study involved the integration of vancomycin, a commonly used antibiotic, into the shell of MBs for the targeting of S. aureus biofilms [41].

Non-targeted treatment approaches combining MBs and US have demonstrated the ability to kill bacteria and remove biofilm [42–44]. It has been demonstrated that MBs can be used as a method for disrupting biofilms in vitro. For example, Lattwein et al. used a combination treatment comprised of MBs, ultrasound and oxacillin to treat an in vitro model of a blood clot-associated S. aureus biofilm [45]. They found that the combination treatment achieved greater efficacy for biofilm remediation than either treatment alone.

Affimers are small (~12 kDa), heat-stable binding proteins that are being developed as replacements for antibodies (~150 kDa) for a wide variety of applications [46]. Affimer proteins, as shown in Fig. 1, have a conserved cystatin scaffold (blue) and two hypervariable loops (purple), the amino acid sequence of which dictates the affinity of an Affimer to its desired target. In addition, due to their smaller size, Affimers tend to be more structurally stable than their antibody counterparts.

In this study we first determined the binding efficacy of the Affimer ACIfA1 using a combination of isothermal titration calorimetry and surface plasmon resonance. ACAIfA1 targets clumping factor A (CIfA) a cell wall bound virulence factor located on the surface of S. aureus cells. CIfA is one of several cell wall-anchored virulence factors that is associated with cell-surface attachment interactions that aid early colonization by bacterial cells and enables the development of biofilms [48–51]. The ACIfA1 Affimer was compared against a control Affimer, Affimer Alainine Cystatin (AAC) which was identical except for removal of the active targeting peptide sequence. We subsequently attached the Affimer to polymer beads and microbubbles, using the NTA/his-tag chemistry, and then determined the efficacy of binding the Affimer labelled polymer particles and microbubbles to a S. aureus biofilm, grown in a microfluidic platform, using a combination of confocal

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**Fig. 1.** A) Crystal structure of the general Affimer protein construct [47]. B) Cartoon representation of the Affimer CIfA1 (ACIfA1), with the hypervariable loop binding site in pink and conserved cystatin scaffold in blue. C) Cartoon representation of the Affimer protein, Affimer Alainine Cystatin (AAC), was used as a control containing a series of alanine repeats in the hypervariable loop sections to prevent specific binding but maintain the cystatin scaffold structure. Both proteins have a poly-histidine tag for purification and bead conjugation, and a cysteine residue for MB conjugation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
fluorescence laser scanning microscopy (CLSM) and bright field imaging.

2. Materials and methods

2.1. Microfluidic platform for biofilm studies

Microfluidic devices (Fig. 2A, Figure S11) similar to those used by Paquet-Mercier et al. were used for biofilm cultivation and subsequent Affimer protein binding studies [52]. Each device contained three chambers, which could be exposed to different control or experimental conditions. Microfluidic devices were fabricated using standard photolithographic techniques. A 3-inch diameter silicon wafer (PI-KEM Ltd, Tamworth, UK) was spin-coated onto the wafer to a height of 180 μm. Designs were then written in the SU8 layer using a 375 nm laser (MicroWriter ML, Durham Magneto-Optics, Durham, UK). Devices were cast from the SU8/silicon master in polydimethylsiloxane (PDMS) using a 1:10 ratio of the cross-linker to polymer base (Sylgard 184, Dow Inc, Midland, MI, USA). The PDMS was left under vacuum for 30 min to remove air bubbles and placed in an oven at 75 °C for 1 h to set. Individual devices were then cut, hole punched, and bound to #1.5H 24 rows. Designs were then written in the SU8 layer using a 375 nm laser (MicroWriter ML, Durham Magneto-Optics, Durham, UK). Devices were cast from the SU8/silicon master in polydimethylsiloxane (PDMS) using a 1:10 ratio of the cross-linker to polymer base (Sylgard 184, Dow Inc, Midland, MI, USA). The PDMS was left under vacuum for 30 min to remove air bubbles and placed in an oven at 75 °C for 1 h to set. Individual devices were then cut, hole punched, and bound to #1.5H 24 × 50 mm glass coverslips (Thorlabs Inc, Newton, NJ, USA) using oxygen plasma treatment (Zepto, Diener, Ebhausen, Germany). Modelling of the devices using flow rates of 80–250 μL/min predicted that the chamber flow velocities would cause fluid shear forces of the order 0.023–0.072 Nm⁻².

2.2. Microfluidic biofilm cultivation

Microfluidic components were sterilized in a vacuum autoclave (Sun 23L Class B, MingTai, Ningbo, China) at 121 °C for 20 min, the microfluidic device was then assembled and exposed to UV for 30 min and rinsed with 70% ethanol and allowed to dry. The system (Figure S12) was equilibrated with autoclaved brain heart infusion broth (BHI, Sigma-Aldrich, St Louis, MO, USA). The microfluidic device was pre-treated with 0.22 μm syringe filtered human fibrinogen (5 mg mL⁻¹, Sigma-Aldrich, St Louis, MO, USA) to promote bacterial adhesion to microfluidic chamber surfaces [53,54]. System sterility was ascertained before bacterial inoculation by plating out BHI broth previously flowed through the system, onto horse blood agar (Thermo Fisher Scientific, Waltham, MA, USA). The microfluidic device was seeded with an overnight culture of UAMS-1 S. aureus cells at a concentration of the order 10⁸ CFU mL⁻¹ and left static for 2 h at 37 °C to allow bacterial adhesion. BHI media was then flowed through the chambers at ~ 80 μL/min for 24 h, at 37 °C. Biofilms were maintained at 37 °C during growth, staining, and imaging aspects of experimental work. The attachment of beads/MBs and the application of US were carried out at room temperature because the device had to be removed from the heated stage of the microscope, this process took ~10 min.

2.3. Fluorescent staining and biofilm imaging

The cultured biofilms were stained using a LIVE/DEAD cell viability kit (Biotium Inc, Fremont, CA, USA) containing DMAO™ for bead and MB attachment proof of concept work. The stain kit was changed to SYTO™ 9 and Propidium Iodide (PI) (BacLight, Invitrogen, Thermo Fisher, MA, USA), which was found to provide more reliable viability data. The former stain kit was used in tMB attachment studies by combining 5 mM DMAO™ with 1 mL of 0.22 μm filtered 150 mM NaCl solution a 1:8 ratio, this was then diluted to 1 mL and vortexed before use. For cell viability assessment, 1.5 μL of 20 mM PI and 3.34 μM SYTO™ 9 were added to 1 mL of 0.22 μm filtered 0.85% NaCl solution and vortexed before use. Biofilms were incubated with the relevant stain for 20 min. Once stained, the microfluidic device was mounted onto a confocal laser scanning microscope (CLSM) (SP8, Leica, Wetzlar, Germany). Fig. 2B shows the CLSM imaging setup applied using a 100 × oil immersion objective (HC PL APO CS2 FWD 0.13 mm NA 1.4).

2.4. Selection of anti-clumping factor A Affimer (AClfA1) proteins

Recombinantly produced ClfA/ySUMO fusion protein and ySUMO protein were biotinylated using EZ-Link NHS-biotin (Cat. No. 20217, ThermoFisher Scientific, Waltham, MA, USA). In brief, for 100 μL of a 12.5 μM solution of ClfA/ySUMO fusion protein, 0.8 μL of 10 mM EZ-Link NHS-Biotin reagent was added and incubated for 30 min at room temperature. Excess biotin was subsequently removed using Zeba Spin.
desalting columns, 7 K MWCO (Cat. No. 89882/89883, ThermoFisher Scientific, Waltham, MA, USA). Phage display screening was performed with stringent negative selection against sYSUMO protein as previously described with one alteration [55]. In contrast to the published protocol, the negative selection was also performed in the first panning round by immobilizing the pre-pan wells with 1 μg of biotinylated sYSUMO protein prior to the incubation with the Affimer phage library. After three rounds of panning randomly selected clones were tested for binding CIIfA by phage ELISA as previously described (Figure S1E) [56]. CIIfA was prepared as outlined in section 4 of the supplementary information.

2.5. Production of Affimer proteins ACIfA1 and AAC

The binding ability of ACIfA1 was compared to a non-binding Affimer scaffold (AAC) control in which the hypervariable loops had been replaced with alanine repeats (Fig. 1C). Affimer proteins were prepared as described in section 4 of the supplementary information. Plasmids containing the vector for ACIfA1 protein expression were transfected into BL21 GOLD DE3 E. coli cells. Protein expression was induced by using auto-induction media. Proteins were isolated from the cell lysate using nickel affinity chromatography. The Affimer proteins used for targeting microbubbles and MB against biofilms were tagged with Alexa Fluor 647 to aid visualization during CLSM imaging. Alexa Fluor 647 NHS Ester (ThermoFisher, Waltham, MA, USA) was covalently attached to N-terminal amines and primary amine groups associated with lysine residues.

2.6. Isothermal titration calorimetry (ITC)

The interaction between CIIfA and ACIfA1 was studied using isothermal titration calorimetry (ITC) (MicroCal, Malvern Panalytical, Malvern, UK). ACIfA1 and the AAC control were dialyzed into phosphate buffer (50 mM Na\textsubscript{2}HPO\textsubscript{4}/NaH\textsubscript{2}PO\textsubscript{4}, 150 mM NaCl, pH 7.4). The protein was concentrated by centrifugal concentration (Amicon 10 kDa MWCO). ITC binding assays were performed using a Microcal iTC200GE, at 25 °C. The ACIfA1 sample was loaded into the sample cell (200 μL, 20 μM) and the Affimer titrant was loaded into the sample syringe (70 μL, 200 μM). Each titration experiment consisted of a sacrificial injection of 0.4 μL followed by 19 injections of 2 μL. Titration data were analyzed using a one-site binding model in Origin 7 software [57]. CIIfA was prepared as outlined in section 3 of the supplementary information.

2.7. Polystyrene bead – Affimer conjugation

A known volume of 2 μm diameter Nickel (II) nitrilotriacetic acid (NTA) polystyrene beads (10^9 beads mL\textsuperscript{-1}) (micromod Partikeltechnologie GmbH, Rostock, Germany) were pelleted using centrifugation (13,000 × g, 2 min) in a 1.5 mL Eppendorf tube. The water storage solution was removed, and the beads resuspended in phosphate buffer (50 mM Na\textsubscript{2}HPO\textsubscript{4}/NaH\textsubscript{2}PO\textsubscript{4}, 150 mM NaCl, pH 7.4) to the original concentration. The beads were then pelleted by ultracentrifugation (13,000 × g, 4 min) and resuspended in 10 μL of ACIfA1 protein, in phosphate buffer, to give the original bead concentration. The presence of a His-tag on the Affimer protein was essential for conjugation. Protein samples were incubated at room temperature for 3–4 h at a low shear rate, fluorescently labelled samples were wrapped in foil to prevent photobleaching. The beads were then pelleted and the supernatant containing unbound protein removed, and the beads resuspended in buffer. Decanting and resuspension steps were repeated twice to wash beads of unconjugated protein, beads were diluted to a final concentration of 10^8 beads mL\textsuperscript{-1} for biofilm attachment studies.

2.8. Lipid preparation

MBs were prepared from a 95:5 M ratio of dipalmititylphosphatidylcholine (DPPC) (Lipoid, Ludwigshafen, Germany) and 1,2-distearyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)]-2000 (DSPE–PEG2000–Maleimide) (Avanti Polar Lipids, Alabaster, AL, USA). The lipids, initially dissolved in a 50:50 chloroform and methanol mixture, were dried on the walls of a glass vial under nitrogen for 40 min. This was followed by overnight vacuum desiccation to remove any remaining solvent in the lipid sample. The lipids were resuspended in 0.22 μm filtered solution of PBS 1% glycerol (vol/vol) to a final concentration of 2 mg mL\textsuperscript{-1}. Prior to MB production, 10 μL of tetradecafluorohexane (C\textsubscript{14}F\textsubscript{34}) (Sigma-Aldrich, St. Louis, Mo, USA) per 1 mL of lipid solution was added to improve stability and MB lifetime [58].

2.9. Microbubble production and sample quantification

MBs containing DPPC and DSPE–PEG2000–Maleimide (95:5) were produced using a multiplexed microfluidic microspray device, previously described [59]. The lipid solution was introduced at a flow rate of 90 μL min\textsuperscript{-1} and combined with a decaffluorobutane (C\textsubscript{6}F\textsubscript{10}) gas stream using a pressure of 1100 mbar. Following production, samples were removed and diluted 10 × in PBS for optical microscopy with a 40 × objective (90X, Nikon, Japan) performed using a 50 μm observation chamber. MATLAB (The Mathworks Inc, Natick, MA, USA) was used to analyze the MB samples and obtain sizing and concentration data [60].

2.10. Microbubble – Affimer attachment and washing

Both AAC and ACIfA1 were attached to the surface of the lipid-shelled MBs via a maleimide-thiol linkage (Michael reaction). A free thiol group was present in the cystatin scaffold of both Affimer proteins (shown in Fig. 1B and C), and maleimide-labelled lipids were incorporated into the MB shell. Following production and initial imaging of MBs, Alexa Fluor 647 tagged ACIfA1 and AAC, both maintained in PBS (pH 7.4), were added to separate MB samples, mixed gently, and incubated at room temperature for 30 min to allow binding. The volume of 7.5 μM Affimer required for each sample was calculated based on the number of MBs in each sample and the maximum packing efficiency of the Affimer. The excess of fluorescently labelled Affimer in solution was removed via a two-step centrifugation wash process, applying a relative centrifugal force of 300g for 10 min, PBS (pH 7.4) was used as the wash buffer. Prior work showed this method to significantly reduce the background fluorescence level whilst preserving the MB population for use. MB samples were imaged as described above using bright field microscopy, typical post-wash concentrations were in the range of 1–7 × 10^8 mL\textsuperscript{-1} with an average sizing of 2.5 ± 0.3 μm. MB samples were diluted to a concentration of 1 × 10^6 MB mL\textsuperscript{-1} for use on-chip.

2.11. Affimer – microbubble (and Affimer – bead) interactions with biofilms

Following biofilm cultivation and live/dead staining, the biofilms were washed for 5 min with HEPES buffer (150 mM NaCl 50 mM HEPES, pH 7.4) to remove any unbound fluorescent stain. A flow rate of 250 μL min\textsuperscript{-1} was used in all subsequent steps. MB (or beads) suspended in phosphate buffer were flowed into the chamber being tested at a concentration of 1 × 10^8 mL\textsuperscript{-1}, the confocal microscope was used to observe the passage of the particles under investigation into the chamber. The flow was then stopped for 10 min, to allow MB binding to the biofilm, in an inverted chamber. The chamber was then re-orientated and washed with phosphate buffer for 30 min. The inversion step was omitted for bead experiments, which have approximately the same density as water. The flow was stopped during imaging and z-stacks acquired using CLSM. Fig. 2A shows a schematic of the microfluidic platform used, isolation of each chamber was controlled via off-chip valves. Z-stack image sets (35 slices measuring 30 μm) were acquired at 20 positions within the growth chambers for bead experiments, and 15 positions for MB experiments. The number of image sets taken was...
2.12. Biofilm viability and analysis

Biofilm viability was assessed using a bacterial viability kit (BacLight™, ThermoFisher Scientific, Waltham, MA, USA) prepared by combining 1.5 μL of SYTO™ 9 and propidium iodide (PI) stain components with 1 mL of 0.22 μm filtered 0.8% NaCl solution. This was incubated for 20 min with the biofilm before being washed with HEPE buffer for 10 min (250 μL min⁻¹, ~90 refreshes of the device) to remove excess stain. CLSM imaging was performed using a 100 × objective. In all cases, the central chamber of the device was imaged as a control, with US/TMB/US + tMB applied to the side chambers. A first round of imaging was performed at time T₀ at 10 locations in each chamber within an area that could be covered by the US beam. This set of initial locations were chosen based on notable markers nearby which ensured that the same positions could be re-found and accurately re-aligned during the second round of imaging (T₁). After US exposure, biofilms were re-stained following the same protocol described above to highlight changes in the viability between image sets obtained at T₀ and T₁. Imaging performed at T₁ (after treatment) was performed in the same locations as before to allow direct comparisons to be made. The elapsed time of experiments was approximately 4–5 h, as measured from the start of the first stain until the final image had been taken.

The live and dead cells visible in fluorescent images were counted manually by overlaying a grid and counting cells in each 12 μm × 12 μm square. The cell counts across all grid squares were summed to give a total cell count for the image. Manual counting was checked in a double blind method on sets of images in different treatment types and were found to be within 10% of each other. The count values were deemed to be not accurate to more than 10%. The dead cell counts (PI stained) were subtracted from the live + dead (SYTO™ 9 stained) cell counts to give separate live and dead cell populations. Changes in the control chamber were counted and used to account for any spontaneous biofilm changes over time. To identify the effectiveness of the different experimental conditions, a fractional change value was calculated for live and dead cell counts for each experimental condition (including the control) (T₀, LIVE/DEAD – T₁, LIVE/DEAD/T₀, TOTAL). This value was calculated for each T₀ and T₁ image pair taken at identical imaging positions. The fractional changes in values were then averaged for the 10 imaged locations and an intra-experimental error was calculated. This was repeated for 3 separate experiments and inter-experimental errors estimated. A student’s unpaired t-test was used for statistical analysis between the different experimental conditions. No statistical differences were identified between first and final locations within the same biofilms, this ruled out cytotoxicity effects contributing to the observed cell death.

3. Results

3.1. Production of S. aureus biofilm under flow conditions

*S. aureus* biofilms were grown in microfluidic devices (Figs. 2A, 2B, S11) at 37 °C under flow conditions chosen to mimic the situation in intravascular infections such as infective endocarditis, where biofilms grow in the bloodstream. A flow rate of 80 μL min⁻¹ was used during the growth phase with wash and MB injection flow rates of 250 μL min⁻¹ [53, 62]. Fig. 2C shows a CLSM fluorescent image of a typical biofilm, grown for 24 h. The cell density shown in Fig. 2D was similar to that found across all three chambers, with lawn-like growths accompanied by larger projecting 3D structures of variable shape/size, as also found by others [63,64].

3.2. Affimer screening and selection

An Affimer phage display library was screened against biotinylated ClfA/ySUMO protein onto streptavidin-coated microwells to isolate specific binders. After three rounds of panning, 48 randomly picked clones were tested for binding ClfA/ySUMO as well as ySUMO using phage ELISA (Figure S13). Clones that confirmed binding to ClfA/ySUMO, but not ySUMO, were sent for sequencing. Sequence analysis revealed 16 unique ClfA specific Affimer clones. Additional screening of these Affimer proteins against *S. aureus* biofilms led to the Affimer designated ACfA1 being selected for further investigation.

3.3. Characterization of the binding kinetics of ACfA1 to clumping factor A (ClfA)

Isothermal titration calorimetry (ITC) was used to assess the binding of ACfA1 and AAC against the clumping factor A (ClfA) target. The equilibrium dissociation constant, Kᵦ, gives an indication of binding affinity [65,66]. The interaction between ACfA1 and its target (ClfA) is shown by the steep curve in Fig. 3A. This reaction gave a dissociation constant (Kᵦ) of 62 ± 3 nM, suggesting a high binding affinity. In contrast, no binding was observed between the control AAC and ClfA, as indicated by the flat line in Fig. 3B. As a result of this, a Kᵦ value could not be calculated for AAC. This result indicates that the interaction between ACfA1 and ClfA is specific to the hypervariable loops in the ACfA1 protein structure.
3.4. AClfA1 and AAC functionalized micro-bead binding to biofilms

AClfA1 and AAC, labelled with Alexa Fluor A647, were attached to 2 μm polystyrene beads (micromod Partikeltechnologie GmbH, Rostock, Germany) and flowed through microfluidic chambers containing S. aureus biofilm. Images were obtained via CLSM, and the number of beads bound to the biofilm surface was counted manually, as described in the methods. The concentration of beads bound to the biofilm per unit area was calculated.

Fig. 3. Isothermal titration calorimetry analysis of AClfA1 and AAC binding to clumping factor A (ClfA). Top traces show the heat flow per injection pulse over time. The raw data are the difference in power supplied to the sample and reference cell to maintain isothermal conditions. Lower traces show the integrated heat change as a function of Affimer addition. The molar ratio is defined as the [Affimer]/[protein] ratio. A) Titration of Affimer AClfA1 into ClfA. B) Titration of Affimer AAC into ClfA.

Fig. 4. A) Bar graph showing the mean number of beads per mm² for AClfA1 and AAC conjugated 2 μm polystyrene beads, bound to S. aureus (UAMS-1) biofilms, after washing with buffer for 30 min to remove unbound particles. Error bars show the standard error obtained from n = 60 images per bead type obtained from three experimental repeats (20 images per repeat). P values indicate: **** ≤ 0.0001. B) Beads conjugated with Affimer AAC-A647. C) Beads conjugated with AClfA1-A647. The green signal shows bacterial cells stained with DMAO™, and the red signal shows beads with Alexa Fluor 647-labelled Affimer attached to their surface. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
area (Fig. 4) was significantly higher (P ≤ 0.0001) for AClfA1 (6211 ± 426 beads mm⁻²), compared to the control AAC (684 ± 99 beads mm⁻²).

3.5. AClfA1 and AAC functionalized MB binding to biofilms

AClfA1 and AAC were attached to lipid-coated MBs, consisting of DPPC:DSPE-PEG2000–Maleimide (95:5) shell and a C₄F₁₀ gas core, and are referred to as targeted MB (tMB) and control MB (cMB) respectively. Fig. 5A shows a significant increase in tMB binding (1390 ± 45 MB mm⁻²) to S. aureus biofilm. Fig. SI 6 shows the spread of binding data for both MBs and beads decorated with AClfA1 or AAC.

3.6. Effect of targeted microbubbles and ultrasound exposure on S. aureus biofilms

Ultrasound (US) induced cavitation effects are known to porate mammalian cells and lead either directly to cell death, or indirectly via enhanced therapeutic uptake [25,43,67,68]. Gram-positive bacteria, however, have an ~60 nm thick bacterial cell wall to protect the cells from lysis and osmotic shock. Thus, it was of interest to understand whether the cavitation of bound MBs would also lead to poration of bacteria or whether it would lead to a break-up of the biofilm, or a combination of these. To assess the effect of bursting the tMB, the biofilms were stained with SYTO™ 9 (green, live/dead cells) and PI (red, dead cells) and imaged before treatment of the biofilm (time = T₀) with US. An ultrasound destruction pulse sequence (2 s, 2.25 MHz, 1 kHz PRF, 22 cycle sine wave, mechanical index of 0.54, 1% duty cycle) was used to burst the MB and the remaining biofilm was re-stained with SYTO™ 9 and PI to highlight changes to cell state and biofilm mass (time = T₁). Representative images from such experiments are shown in Fig. 6. From left to right, columns show images for untreated and US with tMB treated biofilms. After a single exposure combining ultrasound with targeted MBs, a 25% reduction in live cell counts was observed associated with removal of biomass and a concomitant 8% increase in dead cell count. As shown in figures SI6 and SI7, biofilms exposed to US alone or targeted MBs alone did not show comparable levels of cell death.

4. Discussion

4.1. Microfluidics for biofilm study

The microfluidic approach to biofilm study offers an improvement over traditional static systems, such as well plates, as they allow better control over the microenvironment and offer a better mimic of the bloodstream environment where infections, such as infective endocarditis, may occur [45]. Furthermore, microfluidic devices may be easily integrated into existing microscopy systems, thereby allowing non-invasive visualization and imaging in real time of reagent-biofilm interactions. The development of a triple chambered device improved experimental throughput compared to single chamber devices used by others [43,62,69]. CFD modelling of the devices ensured a near-identical growth environment in each chamber through careful flow balancing. By further modifying devices to include separate reagent channels leading into the two side chambers, we were able to apply and study different reagents within the system in parallel. Figure SI1 shows the device used and the associated dimensions, and Figure SI2 shows the whole system including other associated components.

4.2. AClfA1 and ClfA binding kinetics

The AClfA1 – ClfA binding strength, 0.62 nM, was of a similar order of magnitude to that found for other proteins and monoclonal antibodies (mAbs) known to bind to S. aureus which typically display Kᵢ values in the range of 0.47 nM–10 pM [66,70]. In contrast, the Kᵢ values observed between gram-positive bacteria and vancomycin, an antibiotic

![Fig. 5. A) Bar graph showing the average number of MB per mm² for AClfA1 and AAC conjugated MBs following incubation with S. aureus (UAMS-1) biofilms. Measurements made after washing with phosphate buffer for 30 min to remove unbound MBs. Error bars show the standard error obtained from n = 45 images per MB type obtained from three experimental repeats (15 images per repeat). **** = P value ≤ 0.0001. B) MBs conjugated with AAC-A647. C) MBs conjugated with AClfA1-A647. The green signal shows bacterial cells stained with DMAO™, and red signal, MBs with Alexa Fluor 647-labelled Affimer attached to their surface. White circles have been added to highlight areas of MB attachment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)]
commonly used in the treatment of serious infections such as MRSA, ranged from 100 to 1 μM, suggesting that the AClfA1 offers a stronger binding affinity than vancomycin as used by others [41, 71]).

4.3. AClfA1 and AAC binding when conjugated to beads and microbubbles

The approximate 9-fold increase in bead concentration at the biofilm surface mediated by AClfA1 coated beads confirmed that the binding interaction was strong enough to anchor ‘carrier’ particles to the biofilm and withstand an estimated flow-induced shear stress of 0.072 N m\(^{-2}\) at a flow rate of 250 μL min\(^{-1}\).

We noted an approximately 5-fold decrease in the number of tMB attached to the surface compared to their bead counterparts, ~1390 ± 45 MB mm\(^{-2}\) compared to 6211 ± 426 microbeads mm\(^{-2}\). Figure S16 shows the spread of bead (A) or MB (B) counts found for each z-stack image series in an imaging area of approximately 116 μm × 116 μm. The typical length of time the biofilm/MB interactions were studies for was ~2–3 h. Hence, we ascribed these differences potentially to i) intrinsic finite lifetime of MBs, ii) reduction in MBs reaching the target sites due to MB destruction during injection into the microfluidics system, and iii) the increased complexity of the experimental method required to enhance MB/biofilm interactions (e.g. chamber inversion process) [58]. Despite the difference in absolute counts, both Figs. 4A and 5A show a comparable ~9-fold increase in the AClfA1 tMBs/beads compared to the non-targeted AAC control isotypes.

4.4. Effect of microbubble destruction on biofilm viability

We observed a ~25% reduction in the number of live cells residing in the biofilm following exposure to tMBs with US. In comparison, Jc et al., and Agarwal et al., who also utilized MB destruction, observed biofilm biomass reductions of 25–79% [68, 72] in the first study, MBs were applied at concentrations of up to 5 × 10\(^8\)/mL, with US applied at 80 kHz using duty cycles up to 100%. In the second study, a continuous perfusion of MBs was applied for 15 min with 2 s US exposures every 2
incorporate several different approaches to assess the response of biofilm viability following different exposure types could be identified alongside AClfA1. Finally, the methods used to assess the biomass or surface clearance of ~15% compared to the various controls (Fig. 6 and Figure S17 and S18). Whilst not the focus of this proof-of-principle Affimer targeting study, biofilm removal and bacterial cell death may both be enhanced using targeted MBs over untargeted MBs and could potentially be optimized in the future [42,44,68,73]. Complementary quantification methods such as CFU counting were considered to reaffirm the biomass removal data. However, such methods were considered to be incompatible with the microfluidic system used here.  

4.5. Limitations of the study

We have examined biofilms under flow conditions with the specific aim of examining bound MB behavior under flow, rather than using flow to influence biofilm formation per se. This is because the proposed tMB would be injected into the bloodstream and would interact with any intravascular biofilm under conditions of flow. A simple in vitro model cannot replicate the wide variation in flow rates and shear forces that occur throughout the cardiac cycle and within the different elements of the human vasculature, particularly in patients with heart valve disease and/or bloodstream infection. In the setting of infective endocarditis where vegetations are usually found on heart valves or intracardiac devices, the blood flow rate will be approximately the same as blood flow through the heart, but will be variable around abnormal anatomy and devices. Hence, shear forces in these regions will also be highly variable. We estimated the shear forces likely to be produced in our model to allow comparison with other studies. Although this work provides proof of principle that tMB can bind S. aureus biofilms and withstand some degree of shear force, it is likely that an animal model of infective endocarditis would be needed to test the performance of tMB in the varied flow conditions that occur in vivo. Testing the behavior of bound tMB under higher flow rates was not possible because higher flow rates caused biofilm detachment. Testing at lower flow rates was prevented by blockages that formed at lower flow rates. This study has focused on attachment to a single S. aureus strain and it cannot be assumed that the UAMS-1 strain used here is representative of all S. aureus strains. We are currently investigating the affinity of the tMBs of this study against a range of other S. aureus strains and bacterial species. Further investigations are also being conducted to measure the binding affinity of a multitude of other novel Affimer proteins that were identified alongside AClfA1. Finally, the methods used to assess the changes in biofilm viability following different exposure types could be further improved to reduce the observed variability. Future studies will incorporate several different approaches to assess the response of biofilms to different exposure types. These methods could include a combination of growth environments so that bacterial counting, biomass staining, and live/dead staining could all be applied.

5. Conclusions

This work has identified a novel Affimer protein, AClfA1, which can provide a strong level of binding \(K_D = 62 \pm 3\ nM\) against clumping factor A, a virulence factor expressed by S. aureus. We have demonstrated that surface functionalization of “carrier” particles, namely polystyrene beads and lipid-coated MBs showed improved binding to S. aureus (UAMS-1) biofilms by 8–10 times, as compared to a control Affimer protein, AAC. Finally, we demonstrated that the US induced destruction of biofilm-bound tMBs leads i) removal of biofilm and ii) poration of the bacterial cell wall and cell death. However, this requires optimization before being considered a biomechanical method of biofilm treatment or to be used in combination with drugs to improve treatment. Clinical use of this approach would likely involve co-delivery of antimicrobial agents to improve treatment efficacy, as suggested by others [43,45,68].

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Data availability statement

The datasets generated for this study can be found in the University of Leeds data repository, available at https://doi.org/10.5518/774.

CRediT authorship contribution statement

Jack A. Caudwell: performed experimental work, Formal analysis, Writing – original draft, † these authors contributed equally. Jordan M. Tinkler: performed experimental work, Formal analysis, Writing – original draft, † these authors contributed equally. Ben R.G. Johnson: helped with experimental setup design, fabrication of components, Writing – original draft. Kenneth J. McDowall: performed the initial identification and isolation of the Affimer proteins used in this work, Writing – original draft. Fayez Alsulaimani: performed the initial identification and isolation of the Affimer proteins used in this work, Writing – original draft. Darren C. Tomlinson: performed the initial identification and isolation of the Affimer proteins used in this work, Writing – original draft. Christian Tiede: performed the initial identification and isolation of the Affimer proteins used in this work, Writing – original draft. Steven Freear: Conceptualization, Project administration, interpretation, Writing – original draft, gave approval to the final version of the manuscript. W. Bruce Turnbull: Conceptualization, Project administration, interpretation, Writing – original draft, gave approval to the final version of the manuscript. Stephen D. Evans: Conceptualization, Project administration, interpretation, Writing – original draft, gave approval to the final version of the manuscript. Jonathan A.T. Sando: Conceptualization, Project administration, interpretation, Writing – original draft, gave approval to the final version of the manuscript.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biomolbio.2022.100074.

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