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Abstract

The ability to selectively target specific biofilm species with antimicrobials would enable control over biofilm consortium composition, with medical applications in treatment of infections on mucosal surfaces that are colonized by a mixture of beneficial and pathogenic microorganisms. We functionalized a genetically engineered multimeric protein with both a targeting moiety (biotin) and either a fluorophore or a photosensitizer (SnCe6). Biofilm microcolonies of Aggregatibacter actinomycetemcomitans, a periodontal pathogen, were targeted with the multifunctional dodecamer. Streptavidin was used to couple biotinylated dodecamer to a biotinylated anti-A. actinomycetemcomitans antibody. This modular targeting approach enabled us to increase the loading of photosensitizer onto the cells by a cycle of amplification. Scanning laser confocal microscopy was used to characterize transport of fluorescently tagged dodecamer into the microcolonies and targeting of the cells with biotin-labeled, fluorescently tagged dodecamer. Light-induced activity of the targeted photosensitizer reduced the viability of A. actinomycetemcomitans biofilm, as indicated by membrane permeability to propidium iodide. The functionalized multimeric protein promises to be a useful tool for controlling periodontal biofilm consortia and offers a modular design whereby moieties that target different species can be readily combined with the functionalized protein construct.
Targeted delivery of a photosensitizer to *Aggregatibacter actinomycetemcomitans* biofilm

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Abbreviations: chlorhexidine digluconate (CHG); *Listeria innocua* dodecamer (LiDps); LiDps with the KLFC tetrapeptide (KLFC-LiDps); reactive oxygen species (ROS); photodynamic therapy (PDT); monoclonal antibody against *Aggregatibacter actinomycetemcomitans* (anti-Aa antibody); biotinylated KLFC-LiDps (KLFC-LiDps-B); KLFC-LiDps-B conjugated to Alexa Fluor 488 (KLFC-LiDps-B-AF); KLFC-LiDps-B conjugated to SnCe6 (KLFC-LiDps-B-SnCe6)

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Abstract

The ability to selectively target specific biofilm species with antimicrobials would enable control over biofilm consortia composition, with medical applications in treatment of infections on mucosal surfaces that are colonized by a mixture of beneficial and pathogenic microorganisms. We functionalized a genetically engineered multimeric protein with both a targeting moiety (biotin) and either a fluorophor or a photosensitizer (SnCe6). Biofilm microcolonies of Aggregatibacter actinomycetemcomitans (Aa), a periodontal pathogen, were targeted with the multifunctional dodecamer. Streptavidin was used to couple biotinylated dodecamer to a biotinylated anti-Aa antibody. This modular targeting approach enabled us to increase the loading of photosensitizer onto the cells by a cycle of amplification. Scanning laser confocal microscopy (SLCM) was used to characterize transport of fluorescently tagged dodecamer into the microcolonies and targeting of the cells with biotin labeled, fluorescently tagged dodecamer. Light induced activity of the targeted photosensitizer reduced the viability of A. actinomycetemcomitans biofilm, as indicated by membrane permeability to propidium iodide. The functionalized multimeric protein promises to be a useful tool for controlling periodontal biofilm consortia and offers a modular design whereby moieties that target different species can be readily combined with the functionalized protein construct.

Introduction

Therapeutic tools that enabled targeted delivery of antimicrobials to specific species in biofilms would expand our capability to treat chronic infections associated with mucosal surfaces where complete sterility is not the natural healthy condition. The oral cavity and the gut are prime examples of mucosal surfaces at which the immune system maintains a healthy microbial presence (3, 50). Mucosal surfaces in the oral cavity are non-sterile environments colonized by biofilm
consortia (38). In the gut, some commensals are true symbionts (30, 36) and, similarly, it is hypothesized that oral commensals may aid in maintaining a healthy immune homeostasis (35). The implication is that treatment of oral diseases such as periodontal disease can be made more effective by selective elimination of pathogens. By extension, we may also find that tools that enable specific targeting of biofilm pathogens provide effective treatments for chronic infections of the gut (31).

A class of multimeric proteins that self assemble into highly symmetric quaternary structures (7, 52) show promise for development into delivery vehicles for therapeutic (15, 16, 44) and imaging agents (41, 51). We selected one of these proteins, a relatively small spherical 9 nm diameter dodecamer, originally isolated from Listeria innocua (LiDps) (23), for targeted delivery of a photosensitizer to A. actinomyctemcomitans biofilm. We genetically added a tetrapeptide to the C terminus of each of the 12 monomeric 18k Da subunits of LiDps. Cysteines incorporated into this tetrapeptide were labeled with biotin. Since the C terminus is presented on the exterior surface of the assembled dodecamer these biotins were readily available for binding to streptavidin, a universal coupling protein. A SnCe6 photosensitizer was covalently linked to intrinsic lysines.

Photosensitizers are compounds that produce reactive oxygen species (ROS) upon excitation by light (25). Antimicrobial photodynamic therapy (PDT) has been shown to be effective against oral pathogens (6, 29, 54). SnCe6 has been used previously for targeted antimicrobial PDT (10-12). The primary modes of action of photosensitizers are membrane disruption and, if the photosensitizer is internalized, DNA damage (19). The antimicrobial activity of ROS produced by light activated photosensitizers is relatively localized. This is especially the case for singlet oxygen (diffusion length of < 50 nm), which is thought to be the primary ROS causing cell death (32). The localized action of photosensitizers makes them attractive agents for targeted antimicrobial therapy.

The LiDps genetic construct, dual functionalized with SnCe6 and biotin, is a modular unit that can be combined with any targeting moiety that can be biotinylated. This would accommodate a variety of approaches that have been used to target microbial pathogens including...
lectin/carbohydrate interactions (53), antimicrobial peptides (8, 21), and the lysins isolated from bacteriophage (14). We chose to use an antibody as the targeting moiety for these studies since monoclonal antibodies are available for a number of the prevalent periodontal pathogens (5, 9, 17). In addition, antibodies offer a wide range of potential cell surface targets combined with the possibility to engineer high binding affinities (4).

A. actinomycetemcomitans is a gram negative periodontal pathogen implicated as the primary etiological agent in localized aggressive periodontitis (13, 37). Rough colony variants isolated from sites of infection form discrete microcolonies when cultured in vitro which exhibit distinctive cohesive as well as adhesive properties (27). We characterized transport of the LiDps dodecamer to the base of A. actinomycetemcomitans microcolonies, demonstrated targeting of the LiDps to the cells in the microcolonies and showed that SnCe6 targeted to 24 h biofilms produced light activated membrane disruption. The modular design enabled us to use cycles of alternating exposure to the streptavidin and the LiDps dodecamer to increase the loading of targeted photosensitizer onto the cells.

Methods

Bacterial strain and culture conditions

A. actinomycetemcomitans (formerly Actinobacillus actinomycetemcomitans) strain D7, a rough colony clinical isolate obtained from the central incisor of an African American female patient with generalized aggressive periodontitis, was provided by Casey Chen, University of Southern California. The liquid medium was modified tryptic soy broth (MTSB) consisting of (per liter): 30 g tryptic soy broth and 3 g yeast extract. Solid medium was MTSB with 50 ml fetal bovine serum (HyClone) and 15 g bacto agar added (per liter). Frozen stocks were maintained at -80°C in 20% glycerol, 80% MTSB. Biofilms were cultured in 96 well microtiter plates with glass bottoms (MatTek Corp., P96G-1.5-5-F) to enable confocal laser scanning microscope (CLSM) images to be
acquired. The biofilm inoculum was prepared by looping single colonies, cultured on solid medium at 37°C in 5% CO₂ for 72h, into a 1 ml aliquot of liquid medium and dispersing the suspension of cell aggregates by repeated pipetting followed by vortexing and finally diluting the suspension to obtain approximately 10⁷ CFU/ml. No attempt was made to isolate single cells from cell aggregates as described by Kaplan (27). Biofilms were cultured at 37°C in 5% CO₂. Medium was replaced at 24 h for biofilms cultured for 48h.

**Antibody**

Monoclonal antibody (Aa-mAb) 225AA2 (18) against *A. actinomycetemcomitans*, isotype mIgG2b, was purified by affinity chromatography on an AffinityPak™ Protein A column (Thermo Fisher Scientific) following the manufacturer’s recommendations with slight modifications: the wash buffer was Dulbecco’s PBS, pH 8.2 and the elution buffers were 0.1M sodium citrate at pH 5.0, 4.0 and 3.0. Fractions eluted from the column were concentrated to an OD₂₈₀ of approximately 0.2 and dialyzed into 50 mM HEPES (150 mM NaCl, pH 7.0). Purity of Aa-mAb was confirmed by SDS-PAGE under reducing conditions to separate the light and heavy chains. Aa-mAb was biotinylated by reaction for 1 h at room temperature with 0.5 mM of a succinimidyl ester of biotin incorporating a hydrophilic spacer (NHS-PEG₄-Biotin, EZ-Link, Thermo Scientific Pierce, 21362). Unreacted reagent was separated from the Aa-mAb using a BioRad spin column (equilibrated in 10mM Tris, pH 7.4). Immuno dot blots were used to confirm the association of the biotin with Aa-mAb and to check the integrity of the epitope binding site. The covalent addition of the biotin reagent to the light chain of the Aa-mAb was further confirmed by liquid chromatography/electrospray mass spectrometry (LC/MS) as described previously (41). Only the light chain (biotinylated or non-biotinylated) could be detected with LC/MS.

The Aa-mAb 225AA2 epitope has not been characterized at the molecular level. However, its specificity has been characterized (18). The Aa-mAb 225AA2 bound to every *A. actinomycetemcomitans* strain tested (18 strains with 5 different serotypes) and was negative for all
non- *A. actinomycetemcomitans* strains tested consisting of 55 species known to be prominent in the oral cavity (17 genera).

**LiDps mutagenesis and expression**

Addition of residues KLFC to the C-termini of wt LiDps subunit was accomplished by a modification of the QuikChange site-directed mutagenesis (Stratagene) using primers containing extra nucleotides and pET-30b based plasmids containing the wild type LiDps sequence as a template. Primers for mutagenesis contained the nucleotide for the KLFC tetrapeptide (Forward primer: 5' AAGGATCCGAATTCGAGCTCCGAC 3'; Reverse primer: 5' AAGGATCCTAGCAGAATAGCTTTTCTAA GGAGCTTTTCC 3'). The amplified DNAs were transformed into competent *E. coli* strain BL21 (DE) (Novagen). For expression and purification of the assembled KLFC LiDps cultures of the transformed cells were grown overnight at 37 °C in LB/Kanomycin 30 mg/ml. Protein expression was induced with 1.0mM isopropyl-D-thiogalactopyranoside for 5 h. Cells were pelleted at 3700 x g and resuspended in 50mM MES (pH6.5, 100 mM NaCl) with lysozyme (50 μm/ml), RNase (90 μm/ml), and DNase (60 μm/ml). A sequence of sonication and French press treatment were used to lyse cells. Cell fragments were removed by centrifugation at 12000 x g and the supernatant heated to 65 °C for 10 min. The suspension was centrifuged at 12000 x g to remove the aggregated heat sensitive proteins.

Assembled KLFC LiDps was further purified by size exclusion chromatography (SEC) (Superose 6, Amersham Biosciences, Uppsala, Sweden). Identity and purity of the KLFC LiDps protein was confirmed by LC/MS, SDS-PAGE and TEM.

**Functionalization of the KLFC-LiDps**

Purified KLFC-LiDps was biotinylated by reaction of 2 mg/ml protein (0.1 mM protein subunit) with 2 mM of maleimide PEG2 biotin (EZ-link, Pierce, 21901) for 3h at room temperature in MES buffer (pH 6.5, 100 mM NaCl). KLFC LiDps incorporates no cysteine residues except for those added genetically and maleimides react specifically with cysteines of proteins (22).
Assembled dodecamer was purified from the reaction mixture by SEC. Covalent addition of biotin to the protein subunits was confirmed using LC/MS. For transport studies fluorescein was added to the cysteines of the KLFC tetrapeptide using the same protocol but with fluorescein-5 maleimide reagent (Invitrogen). A fluorescent tag (Alexa Fluor 488, Invitrogen) or the photosensitizer (Sn(IV) Chlorin e6, Frontier Scientific, Logan UT) were added to lysines of biotinylated LiDps by reaction with succinimidyld esters. The succinimidyld ester of Alexa Fluor 488 was purchased (Invitrogen, A20000). Carboxylate groups of the succinimidyld ester of the SnCe6 were converted to succinimidyl esters using a modification of a previously published protocol (10). Sn(IV) Chlorin e6 (7 mg), 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydroxycarbamide (3 mg) and N-hydroxysuccinimide (4 mg) were combined in a flame dried glass anaerobe jar which was placed in an anaerobe chamber. To this was added 1 ml of dimethylformamide (dry, stored under nitrogen), the jar was sealed and the mixture was allowed to react for approximately 12 h with mixing. The reaction mixture was aliquoted into 50 µl portions, flash frozen in liquid nitrogen and lyophilized. The succinimidyld esters of the Alexa Fluor 488 or SnCe6 were added to the biotinylated KLFC-LiDps by reaction of 1 mg/ml protein (0.05 mM protein subunit) with 0.5 mM of the reagent for 3h at room temperature in 50 mM phosphate buffer (pH 7.5, 100 mM NaCl). The latter reaction was performed anaerobically in the dark. Prior to addition of the SnCe6 succinimidyld ester nitrogen gas was bubbled through the protein solution for 10 min. The solution was then transferred immediately into an anaerobe chamber, the reagent was added and the mixture was covered with foil and mixed. KLFC-LiDps fluorescently tagged or labeled with SnCe6 was purified by SEC as described above. According to spectrophotometric determination the ratio of labeling of the Alexa Fluor 488 and SnCe6 were both approximately 4 per dodecamer of LiDps . Extensive dialysis did not decrease these ratios. We further measured the extent of non-specific binding of SnCe6 to the dodecamer. Dodecamer was exposed to SnCe6 under identical conditions to those used for reaction with the succinimidyld ester of
SnCe6. In this case co-migration of the absorbance originating from SnCe6 with the dodecamer was negligible.

Transport and targeting of the KLFC-LiDps

Transport of dodecamer into *A. actinomycetemcomitans* biofilm microcolonies and targeting of dodecamer to the microcolonies was characterized using SLCM (Leica TCS-SP2-AOBS). Biofilm microcolonies were visualized through the glass coverslip floor of the wells. This was accomplished by filling each well to the brim, covering the row of wells with parafilm and inverting the plate. Images were collected with either 40X 0.8 NA or 63X 0.9 NA HCX APO L U-V-I water immersion objectives. Green and red fluorophors were discriminated by Acousto-optical tunable filters. Green fluorophors (Fluorescein, SYTO 9 (Invitrogen) and Alexa Fluor 488) were excited with a 488 nm laser, and fluorescence was collected from 500 nm to 543 nm. Red fluorophors (SYTO 59 (Invitrogen) and propidium iodide (Sigma-Aldrich)) were excited with a 561 nm laser, and fluorescence was collected from 600 nm to 721 nm. Images were taken at 1.0 µm intervals throughout the depth of the biofilms. Stacks were combined in Imaris software (Bitplane AG, Zürich, Switzerland) to yield final images. Transport was evaluated by exposing the biofilm to 1 mg/ml KLFC-LiDps labeled with fluorescein and then tracking changes in the fluorescence at the base of the biofilm. Biofilm microcolonies were pre-stained with SYTO 59 for this study so that biofilm microcolonies could be located and brought into approximate focus which required about 3 min after filling the well with the protein solution. Images of the biofilm were acquired at approximately 4 min intervals by repeating the scan using the same parameters. The fluorescence increase in areas at the base of microcolonies was assessed as mean pixel brightness using Adobe Photoshop (7.0). The colonies stained heavily with the SYTO 59 nucleic acid stain resulting in strong red fluorescence and slight bleed through into the green channel. The corresponding pixel brightness of this bleed through was treated as background which was subtracted from the raw data values. Targeting was performed by exposing biofilms to a series of solutions in the wells. Biofilms...
were first washed with 1% BSA in 10 mM PBS (pH 7.0, 100 mM NaCl) for 5 min. Biofilms were rinsed with PBS after each reaction. Solutions and exposure times were: Biotinylated Aa-mAb at approximately 50 μg/ml for 50 min, streptavidin at 50 μg/ml for 30 min and KLFC LiDps at 100 μg/ml for 50 min. The last two steps were repeated to amplify the level of SnCe6 targeted delivery.

Light exposure

SnCe6 was excited with a 633 nm HeNe laser (Melles Griot, Carlsbad, Ca) defocused with a microscope ocular lens. For light exposure the plate was inverted as described above. The plate was placed at a specified distance from the objective so that the area of the beam was slightly larger than the area of the floor of the well. Irradiance (W cm⁻²) was determined using a photometer. The total fluence (J cm⁻²) was determined by the product of the irradiance and the exposure time. The illumination time was 20 min (1,200 s) to achieve a total fluence of 14 J cm⁻² which is in the standard range for antimicrobial PDT (25).

Viability assay

Membrane integrity of A. actinomycetemcomitans cells in biofilm microcolonies was evaluated using the BacLight Live/Dead bacterial viability assay (Molecular Probes) (28). Saline solutions of SYTO 9 and propidium iodide at concentrations recommended by the manufacturer of the kit (L-7012; Molecular Probes) were used. Propidium iodide (red) penetrates cells with compromised membranes while SYTO 9 (green) penetrates all cells. PMT sensitivity for the red and green channels was optimized so that signal was well above background while minimizing bleed through. These optimized settings were used for each experiment. Red/green ratio was quantified as the ratio of pixel intensities of the red and green channels evaluated using MetaMorph software (Molecular Devices Corporation, Downingtown, PA). Microcolonies were selected for analysis, excluding dark regions between the colonies. This was accomplished by "thresholding". Histograms of number of pixels versus pixel brightness were constructed and then truncated to omit the dark
inter-colony regions. Mean pixel brightness of the associated truncated histograms was then calculated. The relationship between the red/green ratio and membrane disruption was assessed by using the red/green ratio induced in biofilms exposed 0.1 mg/ml chlorhexidine digluconate (CHG) for 1h as an internal standard for each experiment. In preliminary studies we determined that exposure of cell suspensions of *A. actinomycetemcomitans* used for biofilm inocula to 0.1 mg/ml chlorhexidine digluconate for 1h reduces CFU by 3 orders of magnitude. (The rough colony *A. actinomycetemcomitans* variant remains primarily in cell aggregates even when dispersed into liquid).

**Statistical analysis**

Efficacy of the targeted SnCe6 was evaluated by a paired t test. Three independent replicates were performed on separate days. Each experiment included 3 or 4 untreated biofilms with the wells for these biofilms distributed along a row of ten wells. Two targeted biofilms exposed to light as well as one well with a non-targeted biofilm exposed to light were also included in each experiment. These wells were located towards one side of the row to minimize the effect of scattered laser light on unexposed wells. Red/green ratios were normalized to the red/green ratio obtained for internal standard (the 0.1 mg/ml CHG condition). Means of within experiment replicates were used for the paired t test.

**Results**

**Transport of LiDps into *A. actinomycetemcomitans* biofilm microcolonies**

Transport of KLFC LiDps into *A. actinomycetemcomitans* microcolonies was characterized using SLCM. The absence of targeting allowed transport kinetics to be measured without the additional factor of binding kinetics. The well was filled with a solution of KLFC LiDps, fluorescently tagged via the peptidyl cysteine residues, and the time course of increase in fluorescence was measured at locations at the base of microcolonies (near the substratum), and in
the interior of microcolonies with respect to the view in the plane of the substratum. The actinomycetemcomitans biofilm was stained with a red nucleic acid stain prior to the transport study (Fig.1A). Fig.1B shows the mean kinetics of transport of the fluorescently tagged KLFC LiDps to locations at the base of 16 A. actinomycetemcomitans microcolonies. Fig.1B indicates that the mean concentration of dodecamer in the interior regions of A. actinomycetemcomitans microcolonies reached a level of approximately 60% of the fluorescent signal in the bulk (between microcolonies) at 20 min.

Figs. 1C and D are SLCM images taken at a section proximal to the substratum interface and 20 min (respectively) after exposure of the biofilm to the fluorescently tagged KLFC LiDps. Locations where data were acquired to construct Fig.1B are indicated. There was a 20% increase in the fluorescence signal from the bulk solution (between the microcolonies) between the first and second time points (4 and 8 min, respectively) which then remained constant. This is likely due to diffusion of the multimeric protein into a static boundary layer near the substratum, accompanied, perhaps, by non-specific adsorption onto the substratum. The 100% value for fluorescence was taken as the value for the later times points in order to construct the data presented in Fig.1B. One possible reason for the exclusion of fluorescence from peripheral regions of the microcolonies may be that there is a greater density of extracellular matrix in these regions. However, we have no direct evidence for this.

Dual functionalized LiDps for targeting

The 12 terminating cysteines (C) on the tetrapeptides of the KLFC LiDps construct provide ideal attachment sites for targeting moieties since they are displaced from the exterior dodecamer shell (23) by the peptide spacer (KLFC). The biotinylation reagent provides a further extension of the spacer length (3 nm) between the biotin and the dodecamer external surface. The covalent addition of one biotin maleimide functional group to each of the monomeric subunits was confirmed
using LC/MS (Fig.2A and B). Some oxidation of the biotinylated product is indicated by the appearance of small higher molecular weight bands spaced 16 Da apart (26).

Biotinylated KLFC LiDps (KLFC LiDps-B) was further functionalized with either Alexa Fluor 488 (KLFC-LiDps-B-AF) or SnCe6 (KLFC-LiDps-B-SnCe6). KLFC-LiDps-B-AF is an analog of the KLFC-LiDps-B-SnCe6 with Alexa Fluor 488 substituted for SnCe6. Succinimidyl esters of these compounds were added to intrinsic lysines of the dodecamer. Association of these functionalities with the dodecamer was confirmed by co-migration of a distinguishing absorbance band of the Alexa Fluor 488 or SnCe6 with the dodecamer on SEC (Fig.2C and D). Extensive dialysis did not reduce the ratio of functional group to protein (determined spectrophotometrically) indicating that the functional groups were covalently bound to the lysines.

**Targeting of A. actinomycetemcomitans biofilm microcolonies with LiDps**

KLFC-LiDps-B-AF was used to characterize targeting of A. actinomycetemcomitans biofilm since SnCe6 fluorescence was not in an appropriate range for SLCM detection. The targeting approach used for both KLFC-LiDps-B-AF and KLFC-LiDps-B-SnCe6 is illustrated in Fig.3A. Streptavidin was used to couple KLFC-LiDps-B-AF to biotinylated Aa-mAb bound to the biofilm cells. The loading of the fluorophor per Aa-mAb epitope binding site was subsequently increased by repeating cycles of exposure to streptavidin and biotinylated dodecamer, with a consequent amplification in fluorescence signal. The first row of SLCM images in Fig.3 (B, i, ii, iii) are 48h A. actinomycetemcomitans biofilms targeted with KLFC-LiDps-B-AF. The mean fluorescence within 48h A. actinomycetemcomitans biofilm microcolonies was increased by factors of 3.4 and 6.3 for one and two repeated cycles of exposure to streptavidin and KLFC-LiDps-B-AF, respectively (Fig.3 B, ii, iii). Binding to A. actinomycetemcomitans microcolonies was primarily mediated through the specific Aa-mAb/epitope interaction as indicated by the much lower fluorescent signal acquired when the Aa-mAb was omitted from the series of reactions (Fig.3 C, i, ii, iii). There was a slight amount of non-specific binding of the dodecamer to the A. actinomycetemcomitans biofilm.
microcolonies indicated by a fluorescent signal in the green channel which was above background, and which was also amplified by cycles of exposure to streptavidin and KLFC-LiDps-B-AF. The KLFC-LiDps-B-AF fluorescence within microcolonies was lower by factors of 248, 802 and 8 (Fig. 3B and C from left to right, respectively) when Aa-mAb was omitted from the reaction. Images of sections in the plane of the interface and sections taken perpendicular to the interface both indicated that the fluorescence distribution in the microcolonies was non-uniform for 48 h biofilms targeted with dodecamer, with a more dense concentration of fluorescence near the peripheries of microcolonies. In contrast, the fluorescence originating from non-specific interactions (Fig. 3 C) was uniformly distributed throughout the A. actinomycetemcomitans colonies. This is more evident if the images are contrast-enhanced (data not shown). This result suggests that the distribution of fluorescence in targeted A. actinomycetemcomitans microcolonies reflects the Aa-mAb epitope distribution in 48 h A. actinomycetemcomitans microcolonies, rather than originating from hindered transport (consistent with the transport study).

In contrast to 48 h targeted biofilms, 24 h targeted biofilms show a more even uniform distribution of KLFC-LiDps-B-AF (Fig. 3, last row, D, E, and F). The brightness/contrast of these images was optimized (identically) to show the pattern of fluorescence. Fig. 3E shows a magnified view of a microcolony. A comparison of pixel brightness within the two squares showed that fluorescence even within the darkest region of a large microcolony was twice that of the background. Fig. 3F shows the level of non-specific binding of the KLFC-LiDps-B-AF to the A. actinomycetemcomitans microcolonies when the Aa-mAb was omitted.

**Light induced membrane disruption of SnCe6 targeted A. actinomycetemcomitans biofilm**

The red/green color ratio determined from the viability assay upon exposure of biofilms to 0.1 mg/ml CHG for 1 h was used as an internal standard for assessing the efficacy of light induced membrane disruption of SnCe6 targeted cells. The rationale for this was that CHG has been shown previously to be efficacious against A. actinomycetemcomitans biofilms (48) and the primary mode...
of action of CHG is membrane disruption (24). According to the viability assay there was a clear break point between 0.1 mg/ml CHG and 0.01 mg/ml CHG (Supplementary Material, Fig.1S). A. actinomyctetemcomitans biofilms exposed to these two CHG concentrations for 1h were included in each experiment as internal standards.

A. actinomyctetemcomitans biofilms were targeted with KLFC-LiDps-B-SnCe6 using the same protocol as for KLFC-LiDps-B-AF (Fig.3A). In a preliminary experiment we obtained no evidence of membrane disruption of 48 h biofilms. We proceeded to test 24 h biofilms and obtained evidence of membrane disruption for biofilms targeted using two cycles of exposure to streptavidin followed by KLFC-LiDps-B-SnCe6. We attempted to gather data demonstrating a correlation between loss of viability and cycles of amplification using the KLFC-LiDps-B-SnCe6 construct but the variability was too great to make any strong claims. As indicated in Fig.4 membrane disruption induced by exposure of SnCe6 targeted A. actinomyctetemcomitans microcolonies to light (Fig.4B) was intermediate between that of untreated microcolonies (Fig.4A) and microcolonies exposed to 0.1 mg/ml CHG (Fig.4C). The mean red/green ratio for SnCe6 targeted microcolonies exposed to light compared to those exposed to 0.1 mg/ml CHG was 0.62 (SD 0.21) for three independent experiments. The red/green ratio for the SnCe6 targeted microcolonies exposed to light was higher than both untreated microcolonies and SnCe6 targeted microcolonies not exposed to light at the 5% level of confidence (Table 1). The red/green ratio for the SnCe6 targeted microcolonies exposed to light was higher than both microcolonies exposed to light but not targeted with SnCe6 and microcolonies exposed to 0.01 mg/ml CHG at the 10% level of confidence (Table 1). Data for all three experiments are in Supplementary Material (Fig.2S).

Discussion

Our results indicate that the KLFC-LiDps can be transported into A. actinomyctetemcomitans microcolonies during a reasonable time period, that cells within the microcolonies can be targeted
with the dodecamer and that this multimeric protein can be used as a vehicle for targeted delivery of a photosensitizer to *A. actinomycetemcomitans* microcolonies. The susceptibility of *A. actinomycetemcomitans* biofilms to antimicrobials has been shown to decrease as the biofilm matures (45). Consistent with these results, we observed no light induced decrease in viability of 48 h biofilms targeted with photosensitizer, while there was clear evidence of light induced membrane disruption of 24 h biofilms targeted with photosensitizer. The more evenly distributed Aa-mAb epitope distribution in 24 h *A. actinomycetemcomitans* microcolonies may have been partially responsible for this result (Fig. 3). In general, mechanical plaque removal is not sufficient to completely eliminate oral biofilm (2), and this has been shown to be the case for *A. actinomycetemcomitans* (34). Selective killing of *A. actinomycetemcomitans* early stage biofilms would presumably enhance the ability of commensals to compete with *A. actinomycetemcomitans* during recolonization (47), thus conferring a measure of colonization resistance to periodontal tissues (33). In addition, the recolonization by a consortium in which commensals were predominant might aid in establishing a healthy immune balance at diseased sites, allowing periodontal tissues more leverage to eliminate intracellular (invasive) *A. actinomycetemcomitans*.

Transport of macromolecules through a polymicrobial oral biofilm was found previously to be significantly hindered (49). The shape of the data curves presented in Fig. 1B suggests that the LiDps dodecamer attained about 90% saturation in central regions at the base of the *A. actinomycetemcomitans* biofilm microcolonies at 20 min. The fluorescence in these regions at 20 min was about 35 - 95 % of the fluorescence in the bulk, with a mean of about 60%. An extensive review of the literature indicated that the diffusion coefficient of macromolecules the size of the LiDps (molecular weight, 222 kDa) could be reduced in biofilms by as much as a factor of about 0.02 compared to the diffusion coefficient in water (39). This translates to about 17 min to reach 90% saturation in a planar slab 30 µm thick, indicating that the transport kinetics of the LiDps dodecamer that we measured are consistent with previous studies. In terms of eventual clinical
application, the mean fluorescence at 5 min in locations that were relatively inaccessible was approximately 25% of the bulk fluorescence. Estimating the volume excluded by the cells to be 50%, the intercellular concentration at 5 min in the interior portions of the biofilm was about 50% of the bulk concentration. This may be sufficient to effectively target *A. actinomycetemcomitans* biofilms during a 5 min exposure period if the affinity coefficient for binding of the targeting moieties is sufficiently high.

Advantages of the targeting approach we used in this study include modularity and ability to amplify the loading. The modularity of the system allows different targeting moieties to be combined easily with a single functionalized multimeric protein. We have shown previously that loading of a small molecule onto a 12 nm 24 subunit small heat shock protein from *Methanococcus jannaschii* can be increased substantially by incorporating a polymer confined to the interior cavity delineated by the protein shell (1). Although the nucleation and stepwise growth required to synthesize the polymer is fairly sophisticated, the process can be streamlined to produce large quantities of the biotinylated product. Recently, we synthesized an asymmetrically biotinylated LiDps dodecamer that enables one step targeting via an antibody while preserving the modular design (42). Another advantage of our targeting approach is the possibility of increasing the loading with a sequence of applications. Previously, we characterized signal amplification that can be obtained by exploiting the streptavidin/biotin interaction to obtain clusters of the CCMV capsule bound at nucleation sites (40). We acquired AFM images to confirm formation of CCMV clusters at a planar interface (43). Here, we used a similar approach to increase the photosensitizer loading at targeted sites. Although the amplification in the fluorescence signal shown in Fig. 3B suggests that aggregated clusters of the dodecamer are forming at sites of Aa-mAb binding as depicted in Fig. 3A we have no direct evidence to support this interpretation. With respect to clinical relevance, topical application of a sequence of agents may be feasible if the application time is within 5 min. However, for some applications it will obviously be desirable to have a one step administration.
The antimicrobial action of photosensitizers against gram negative bacteria is known to be less than against gram positive bacteria (19, 25). One method to enhance photosensitizer action against gram negative bacteria is to couple the photosensitizer to a cationic peptide or polymer (20, 46). This enhancement may originate from the localized concentration of photosensitizer near the cell membrane, or, alternatively, from disruption of the membrane by the cationic moieties. Since the dodecamer is too large to have entered cells by any known process, the former explanation is consistent with the loss of viability we observed for cells targeted with photosensitizer and exposed to light. We cannot claim that cells in biofilms were in fact killed since we have no direct evidence for this. However, our results indicate that membranes of photosensitizer targeted cells exposed to light were more permeable than controls, and thus these cells may be sufficiently damaged to substantially decrease their competitiveness when growing in a developing oral consortium.

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References


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Figure legends

FIG.1. Transport of fluorescently tagged KLFC-LiDs into *A. actinomycetemcomitans* biofilm microcolonies. A) Biofilm microcolonies stained with SYTO 59 (4.167 µm tick spacing). Microcolonies circled with the dashed lines are the same ones circled in C (below). B) Transport kinetics of fluorescently tagged KLFC-Dps into regions at the base of biofilm microcolonies; The solid line connects data which are the mean of measurements at 16 locations; error bars are the standard deviations. C) Section acquired near the substratum 4 min after exposure of the biofilm to the fluorescently tagged KLFC-LiDs; regions used to acquire the data presented in B are indicated. D) Section acquired near the substratum 21 min after exposure of the biofilm to the fluorescently tagged KLFC-LiDs.

FIG.2. Dual functionalized KLFC-LiDs. A, B) LC/MS deconvolutions of mass spectra of monomeric subunits of the dodecamer. A) mass of non-functionalized KLFC-LiDs monomeric subunits (18540 Da); B) mass of biotinylated KLFC-LiDs (KLFC-LiDs-B) monomeric subunits (19065 Da). The difference in mass between A and B (525 Da) is expected for addition of the biotinylation reagent to the cysteines. The deconvolved spectrum in B indicates that all the 12 subunits of KLFC-LiDs were biotinylated. C, D) Size exclusion chromatograms of biotinylated KLFC-LiDs tagged with Alexa-fluor 488 (KLFC-LiDs-B-AF) (C) or functionalized with the SnCe6 (KLFC-LiDs-B-photosensitizer) (D). The solid lines are the absorbance at 280 nm (protein). The dashed lines indicate the absorbances of the Alexa-fluor 488 (488 nm) or photosensitizer (410 nm) (C and D, respectively). The vertical straight line indicates the position at which the dodecamer elutes.

FIG.3. Targeting of *A. actinomycetemcomitans* biofilm microcolonies with KLFC-LiDs-B-AF. A. Illustration of presumed cluster formation produced by cycles of exposure to streptavidin followed
by KLFC-LiDps-B-AF resulting in an amplification of the fluorescent signal at sites of Aa-mAb binding; Ab: Aa-mAb; StAv: streptavidin; Dps: KLFC-Dps; CWE: cell wall epitope; 1 cycle means Aa-mAb followed by streptavidin followed by KLFC-Dps; B. 48 h biofilm microcolonies targeted with biotinylated KLFC-Dps tagged with Alexa-fluor 488 for 1 to 3 cycles of exposure to streptavidin followed by KLFC-LiDps-B-AF; 1 cycle (i); 2 cycles (ii); 3 cycles (iii); C. Biofilms treated the same as in B except that the Aa-mAb was omitted from the first step. D. 24 h biofilm targeted with biotinylated KLFC-Dps tagged with Alexa-fluor 488; E. magnified view of a selected portion of D, showing the relatively even distribution of fluorescence from the targeted dodecamer; pixel brightness computed for two small regions indicated that even the darkest region of the microcolonies was twice as fluorescent as the background (small squares). F. Biofilm was treated the same as D but the Aa-mAb was omitted.

FIG.4. Membrane disruption of *A. actinomycetemcomitans* biofilm microcolonies targeted with the photosensitizer assessed by the BacLight Live/Dead bacterial viability assay (images of biofilm microcolonies); A) not treated; B) targeted with photosensitizer and exposed to light; C) exposed to 0.1 mg/ml CHG.
Table 1. Statistical level of significance for targeted light induced membrane damage

<table>
<thead>
<tr>
<th>Pair</th>
<th>Relative damage</th>
<th>p value</th>
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</thead>
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<tr>
<td>PSL,NT</td>
<td>3.78</td>
<td>0.0184</td>
</tr>
<tr>
<td>PSL,PS</td>
<td>2.77</td>
<td>0.0286</td>
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<tr>
<td>PSL,L</td>
<td>4.94</td>
<td>0.0995</td>
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<tr>
<td>PSL,CHG(.01)</td>
<td>4.29</td>
<td>0.0904</td>
</tr>
</tbody>
</table>

1 PSL: targeted with photosensitizer and exposed to light; NT: not treated; PS: targeted with photosensitizer, no light exposure; L: exposed to light, not-targeted; CHG(.01): exposed to 0.01 mg/ml CHG

2 Ratio of mean red/green ratios (PSL/paired condition) for three independent experiments

3 Determined by a paired t test for three independent experiments
Figure 1

Time (min)
LiDPS fluorescence (%)
0 5 10 15 20
0
20
40
60
80
100

A

B

D

30 µm

C

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Figure 3
Figure 4