Identification of Specific Target Ligands to Overcome Periodontal Disease Through Phototherapy

DEVELOPING OF TARGETING AGENTS TO OVERCOME BIOFILM THROUGH PHOTOTHERAPY

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Abstract

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Background: Conventional antibacterial treatment fails to eradicate biofilms associated with dental caries and periodontal disease. Biofilm targeted-photodynamic therapy (PDT) could be one of the solutions to these problems. **Objective**: to identify broad-spectrum bacterial binding ligands from drug libraries to develop targeting agents against the biofilm related infections thorough phototherapy.

Material & Methods: Biofilm bacteria of *S. mutans* and *E.coli* were created on tissue flasks. After thorough washing to remove the free planktonic bacteria, the biofilm bacteria were scraped with a sterile blade. The scraped bacteria were ultra-sonicated and were incubated with drug libraries. The beads with *S. mutans* binding were sorted. After removing the bacteria from beads, the *S. mutans* binding beads were then re-incubated with *E.coli*. The sequences were determined by sequence decoding chemistry. The focused library was generated according to the chemical mortif. The bacteria of *S. mutans, P.gingivitis, E.coli, P. aeruginosa, Klebsiella sp, E. faecalis, citrabacter sp.,* and *S. aureus* were screened with focused library one by one. The compound beads with the property of binding to all of above bacteria were isolated and then incubated with human oral epithelial cells as well as human endothelial cells. The beads that bind to bacteria, but not to human oral epithelial cells and endothelial cells were finally identified. The chemical structures of identified compounds were determined by decoding. The bacterial binding compounds were then conjugated with biotin through lysine to create imagine probes and challenged with biofilm to evaluate their imagine efficacy through streptavidin 488 fluorescent fluorophore. The "all in one" targeting nanoparticles with hydrophobic core of photosensitizers were developed by decorating these bacterial binding ligands with porphyrins through click chemistry. The phototoxic efficacy of targeting nanoparticles on biofilm was evaluated by using viable account.

Results: four bacterial binding compounds were identified for possessing the property of specifically binding to all of the above bacteria, but not binding to normal human oral cells and endothelial cells. MTT assays indicated that these four compounds have no cytotoxicity to human epithelial cells and endothelial cells at up to 50 uM. Zonal inhibition assay indicated these four bacterial binding ligands do not have bactericidal effects. Compared with the porphyrin nanoparticles control, targeting nanoparticles might cause a two log fold reduction in the number of mix *E. coli* and *S.mutans* in the biofilm.

Conclusion: A high throughput bacterial binding compound screening method was established, and four novel bacterial binding compounds were identified. The use of a compounds photosensitizer conjugate is a more selective method of delivery thermal and dynamic therapy to biofilm. Work is currently under way to evaluate the phototoxic efficacy of these targeted nanoparticles on *S. mutans, P.gingivitis, E.coli, P. aeruginosa, Klebsiella sp, E. faecalis, citrabacter sp.,* and *S. aureus* biofilm to identify the optimal parameters to fight biofilm.

CHAPTER 1. INTRODUCTION

1.1. Dissertation motivation and clinical significance:

Biofilms are defined as orientated aggregations of microorganisms attached to each other or to a surface and enclosed in extracellular polymeric substance (EPS) produced by themselves (1,2). Furthermore, many biofilms are bathed by some flowing fluid (i.e., water flowing over moss-covered rocks in a stream or saliva flowing over dental plaque on a tooth surface) (3,4). The components of mature biofilm are approximately 5–25% bacterial cells and 75–95% glycocalyx matrix. Biofilm formation is a key virulence factor of a wide range of microorganisms that cause chronic infections. Oral biofilm is unique among the various types of biofilms as it typically requires host salivary glycoproteins to attach to. There are over 700 different bacterial species in the oral microflora. Those species colonize the teeth, tongue, oral mucosa, hard palate, carious lesions, periodontal pocket, et al (5,6). Depending on local ecological factors, the composition of the dental biofilm may vary considerably. With access to excess carbohydrates, the dental biofilm will be dominated by mainly gram-positive carbohydrate-fermenting bacteria causing demineralization of teeth, dental caries, which may further lead to inflammation and necrosis in the pulp and periapical region, i.e., pulpitis and periapical periodontitis (7,8). In supra and subgingival biofilms, predominantly gram-negative, anaerobic proteolytic bacteria colonize and cause gingival inflammation and breakdown of supporting periodontal fibers and bone and ultimately tooth loss, i.e., gingivitis, chronic or aggressive periodontitis, and around dental implants, peri-implantitis (9,10). Biofilms can protect bacteria from a challenging environment with several host defense mechanisms directed towards bacteria or protect from applied antibiotics or bactericidal agents. The antibiotic resistance of bacterial cells in biofilm was reported to be 1,000 to 1,500 times greater than the resistance of planktonic cells (11,12). The antigens of

biofilm bacteria are hidden in the biofilm matrix and become less susceptible to the host immune system. The multifactorial nature of biofilm development and drug tolerance imposes great challenges for the use of conventional antimicrobials and indicates the need for multi-targeted or combinatorial therapy.

Recently, growing attention has been paid to antimicrobial photodynamic therapy (PDT) in dentistry. PDT can be defined as eradication of target cells by reactive oxygen species produced by means of a photosensitizing compound and light of an appropriate wavelength (13,14). It could provide an alternative for targeting microbes directly at the site of infection, thus overcoming the problems associated with antimicrobials. Photodynamic action describes a process in which light, after being absorbed by dyes, sensitizes organisms for visible light induced cell damage PDT has been found to be effective in inhibiting biofilm producing organisms by inducing the production of reactive oxygen species (ROS). Access of the photosensitizer and light to the oral or pocket present no great difficulty, therefore, application of PDT to periodontal infection could prove to be a valuable adjunct to mechanical procedures, if the photosensitizer has broad spectrum activity against bacterial pathogens and selectivity for prokaryotic cells, ie, targeted phototherapy. The use of photosensitizer to produce photothermal and photodynamical cytotoxic affect to bacteria has the following advantages: (I) Bacterial development of resistance to the PDT is less likely to happen as singlet oxygen and other free reactive species created by PDT, interact with several cells structures and different metabolic pathway; (II) As PDT is a non-invasive localized therapy, following application of targeting sensitizer, a light source can be delivered into the target area precisely via a fiber optic cable, so the disturbances of the microflora at other sites would

not occur and the damage to the adjacent host tissues might be avoided. (III) The PDT offers thorough irrigation and elimination of pathogens in inaccessible areas of periodontal packet within short span of time, thus beneficial to both operator and the patients; (IV) The risk of bacteremia after periodontal debridement can be minimized; (V) There is no need to prescribe antibiotics, therefore the possibility of side effect and development of antibiotic resistant strains are avoided. (VI) Furthermore, if this strategy is proved effective in eliminating biofilm bacteria, it can be applied to overcome the bacterial biofilm in medical instruments, such as endoscopy, catheters, etc. (15,16,17). However, up until now, PDT on biofilm was hindered due to a lack of specific targeted ligands to bacteria. The undesired damage to normal cells may be caused due to the nonselective nature of PDT. Therefore, targeted PDT is preferred on one hand to enhance antimicrobial effects and on the other hand to reduce cytotoxicity to normal cells. Highly specific targeting phototherapy technique, if proved effective against biofilm, might highlight the potential of PDT as a promising method to achieve successful disinfection.

This research project involves the discovery of specific biofilm bacterial binding ligands by using combinatorial chemistry strategy. The broad-spectrum bacterial binding ligands will be used to build targeting nano-carriers against biofilm through phototherapy.

Significance:

I: Targeting probe to biofilm bacteria may provide new techniques of microbiology to initially detect biofilm infection in human tissues. Routine microbiological examinations are important and reliable for diagnosis of infections, but somehow less sensitive for biofilm detection (18,19,20). Therefore, new techniques of microbiology on biofilm should be introduced as efficient complements of routine microbiological examination or part of the novel routine methods in hospitals for diagnosis of biofilm infection.

II: Highly selective "all in one" or "on-demand activation" targeting nanoparticles, if proven successful, the new technology can be translated into novel and effective theranostic agents to improve the biofilm diagnosis and treatment.

III: These broad-spectrum bacterial binding ligands might be also conjugated with granulocytes binding ligands, complement components, or fragment of antibody, to induce granulocytes' phagocytosis, thus avoiding antibiotics. Since the ligands discovered here are made of a mix of L and D-amino acids as well as small molecules, they are expected to be more resistant proteinase digestion.

VI: According to the National Institutes of Health, up to 80% of human bacterial infections involve biofilm associated microorganisms. The methodology reported here can be valuable in the search for other biofilm bacteria to construct specific targeting agents against medical device biofilm.

1.2. Rationale for the development of therapeutic agents to overcome biofilm

Targeted molecules have become the goal of biofilm diagnostic and treatment because of its specificity in attacking bacterial cells instead of normal tissues by focusing

on trying to combine targeting molecules with diagnostic imaging agent and/or therapeutics into one single entity, enhancing their site-specific bacterial imaging and delivery while reducing off-targeting agents. More emphasis is now focused on the identification of peptidomimetic molecules instead of monoclonal antibodies to be the better cell surface-targeting agents because they are (a) smaller and therefore might be diffused into biofilm if conjugated with nanoparticles (21,22,23); (b) less likely to bind to the reticuloendothelial system such as liver, spleen, and bone marrow (24, 25); and (c) easy to derivatize chemically. Therefore, peptidomimetic molecules will provide the perfect match for the purpose to the discovery of biofilm biomarkers for the development of the theranostic agent for biofilm infections. One-bead one-compound (OBOC) combinatorial strategy has been proven to be a powerful tool for identification of synthetic ligands against specific biological targets (26) (Figure 1.1)

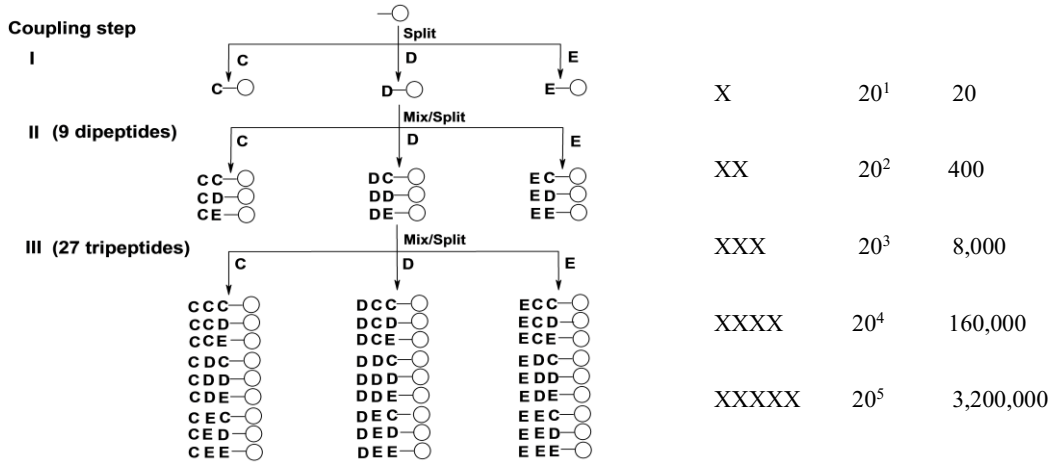

Figure 1.1. Schematic illustration of generation of OBOC combinatorial library.

In this method, the combinatorial library is prepared by a "split-mix synthesis" approach using polystyrene beads (88-micron diameter) as solid support. As a result, each resin bead displays only one chemical entity and there are approximately 10^{13} copies of the same chemical entity on and within the same resin bead. This synthesis feature enables one to rapidly screen hundreds of thousands to millions of library resin beads with an on-bead binding or functional assays. The exact chemical nature of the compounds on selected beads with specific biological function can be determined with an automatic micro-sequencer. This technique has been applied to the discovery of targeting ligands against various biological targets, such as cancer cell surface receptor (27, 28, 29, 30), protein kinase substrates and inhibitors, (31,32) protease substrates and inhibitors(33,34), artificial enzymes, (35, 36), various ligands for the preparation of affinity column media (37) and antibiotics (38).

However, targeted therapy not only needs specific recognition of biofilm cells but also need specific delivery of anti-bacterial or host modulating drugs to biofilm site as well. Hence, nanotechnology is an emerging field that has shown great promise for the development of novel diagnostic imaging and therapeutic agents for a variety of diseases and conditions including bacterial infections. Nanotechnology deals with the design, synthesis and applications of materials ranging in the nanoscale region. The particles with sizes from 1–100 nm are termed as NPs. NPs are preferred over other agents because of their small size, large surface area and highly reactive nature. Their high surface area makes them suitable drug carriers. Targeting compounds can be conjugated on the surface of NPs to increase their solubility and targeted delivery. A few researchers reported that NPs can penetrate and go inside the biofilm structure to destroy bacterial in biofilm (39,40). Recently,

our previous lab has developed several novel nano-carriers for the delivery of paclitaxel (PTX) or other hydrophobic anti-cancer drugs (41, 42, 43). Using a reversible disulfide crosslinked micelle system named nano porphyrins (NPs), (PEG5k-Cys4-CA8 teloderdrimers), hydrophobic drugs can be encapsulated and triggered to be released at the tumor site and inside the cancer cells with high reductive potential. PEG5k-Cys4-CA8 loaded with PTX and decorated with ovarian cancer binding ligands (discovery through OBOC library screening), exhibit superior anti-tumor efficacy and lower systemic toxicity profile in dude mice bearing ovarian cancer tumor xenografts, when compared with equivalent doses to non-targeted PTX nanoparticles as well as clinical PTX formulation (Taxol R) (44). A few researchers reported that recent progress in synergistic chemotherapy and phototherapy by targeted drug delivery systems for cancer treatment (45,46,47).

In this project, we have used combinatorial library technology to identify biofilm specific ligands for the improvement of screening and diagnostic techniques in parallel with the development of new therapeutic molecules against biofilm bacteria.

1.3. Research hypotheses and specific aims

The overall goal of the proposed research is to discover biofilm bacterial binding ligands using OBOC combinatorial strategy to develop targeting theranostic agents against biofilm, including oral biofilm. **Our hypothesis** is that specific ligands against biofilm bacteria might be identified through the high throughput screening of combinatorial libraries. The biofilm binding ligands can be conjugated to optical dyes or nanocarriers to develop theranostic agents, thus enhancing the detection of biofilm infection as well as

the delivering of targeting nanoparticles to biofilm to inactive them through phototherapy while sparing the normal tissue or cells.

The specific **aims** of this project are as follows:

AIM 1: Identification of biofilm bacterial binding ligands from OBOC combinatorial libraries. Twenty-four OBOC combinatorial libraries are generated using split-mix synthesis method. The libraries were incubated with bacteria in small volume to select suitable OBOC libraries for the large-scale screening using live biofilm bacteria cells to identify the compound beads which only bind to different bacteria cells but not bind to normal human cells. MTT assay is used to evaluate potential cytotoxicity of identified ligands. The focused libraries will be generated to search for broad-spectrum biofilm bacterial binding ligands based on binding motif.

AIM 2: Development of biofilm imaging probes and evaluation of their targeting efficacy. Biofilm probes are generated by the conjugation of optical dyes (Q DOTS, fluorescent, IR, organic and etc.) with biofilm targeting ligands that are identified from AIM 1. These imaging agents are evaluated for their binding ability and specificity to biofilm using peptide-histochemistry assay.

AIM 3: Generation targeting theranostic agents.

3.1. Develop "all in one" biofilm bacterial targeting nanoparticles by conjugating biofilm ligand with nano porphyrins. Nano porphyrins (PEG^{5k}-Cys_4-CA_8 telodendrimers) are prepared according to our previously lab's published methods (44). Click chemistry is used for the conjugation of bacterial binding ligands (45).

3.2. Comparison of the biofilm eradiation efficacy of the targeting NPs to that of polymeric NPs in order to elucidate antibiofilm potential. The efficacy of photodynamic and photothermal therapeutic activity of the biofilm targeting nanoparticles will be evaluated on the multispecies biofilm via viable accounts and live & dead staining assay. The schematic summary of the project is shown in Figure 1.2

Create biofilm to obtain biofilm bacteria

↓

screened with OBOC compound libraries

↓

develop focused library based on binding motif

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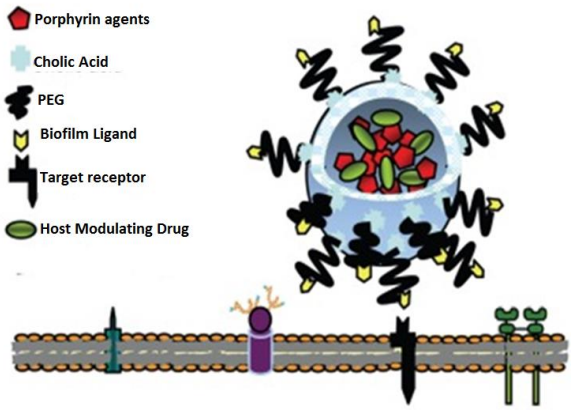
large scale screening to select specific broad-spectrum bacterial binding compounds

↓

develop bacterial biofilm probes to visualize bacterial biofilm

↓

generating porphyrin-based targeting ligand decorated nanoparticles



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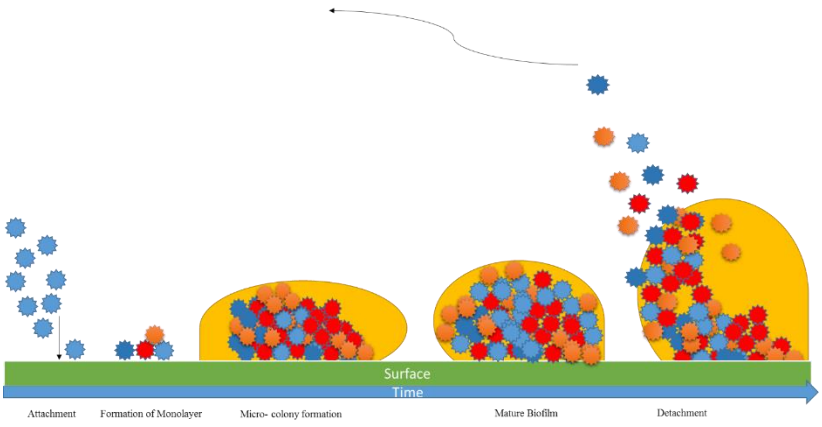
in vitro evolution of therapeutic efficacy of mix biofilm through phototherapy.

Figure 1.2 Schematic summary of the project

CHAPTER 2. Literature Review

2.1. Biofilm architecture, resistance, diagnosis and current therapeutic approaches

A structured consortium attached on a living or inert surface formed by microbial cells attached to each other and surrounded by the self-produced extracellular polymeric matrix is known as biofilm. Biofilms differ from single cells in structure, function, and behavior. Biofilm cells adhere via different receptor–ligand interactions to a surface or to other cells (homotypic or heterotypic); produce EPS; metabolize slowly or rapidly grow or stay attached or disperse (34). Process of biofilm development can be broadly grouped into four different phases as shown in Figure 1.3: attachment (on surface, inanimate or tissue); sessile growth phase governed by intercellular interaction (quorum sensing (QS) factors); biofilm maturation (induced exopolymeric substances (EPS) matrix synthesis) and detachment (induction of disassembly factors) (35).



The circulatory water channels in the biofilm allows for the secretion and exchange of auto-inducers and communication between species (36). Broadly, biofilms comprise of two components, the microbial cells and secreted EPS that constitute 90% of the overall biomass. EPS contributes to overall establishment of biofilm structure and mainly

constitutes of exopolysaccharides that provide sites for cohesion and adhesion interactions, proteins that act as carbon and energy source, and extracellular DNA (eDNA) for resistance gene transmission. Extracellular matrix also modulates biochemical properties thereby regulating diffusion, adhesion and cohesion to create a suitable acidic environment for biofilm formation 5,6). Based on functional divergence EPS can be categorized into three major classes (37). Class I constitutes the architectural EPS that are involved in signal and structural regulation, class II comprises of protective EPS that provide protection against host immune system and physiological stresses and class III that contains aggregative EPS involved in adhesion and biofilm development (37). Microorganisms in biofilms inhabit a matrix that glues them onto inert or biological substrates and provides communal benefits such as increased antibiotic resistance, slow growth, and differential gene expression, elevated levels of lateral gene transfer, stress resistance and subversion of host defense mechanisms. In contrast to upper layers of antibiotic susceptible cells, persister cells are extremely tolerant to antibiotics and occupy the internal core of biofilms. As a result, those biofilm infections are 1000 to 1500 times more resistant to the effects of antimicrobial agents (10). Extensive and unregulated usage of antibiotics has led to development of several antimicrobial resistant strains including methicillin-resistant S. aureus (MRSA), vancomycin-resistant Enterococcus, carbapenem-resistant Enterobacteriaceae and multidrug-resistant Acinetobacter (38). National Institutes of Health (NIH) revealed that 65 – 80% of microbial and chronic infections are associated with biofilm formation and are the *fourth leading cause of death in the U.S.* with 2 million cases annually, which pose challenges in treatment regimens (39). Alarming incidence of antibiotic resistance, unavailability of newer antibiotics and

recalcitrant and chronic properties of biofilm associated diseases demand new sensitive detection and control strategies.

The diagnosis of clinical biofilm-associated infections (BAI) are even more challenging. Routine microbiological examinations are important and reliable for the diagnosis of infections, but somehow less sensitive for biofilm detection. The development of molecular-based diagnostic approaches to BAI is central to the improving of the detection and identification of microorganisms and establishing their role in pathogenesis. Several molecular techniques are now used routinely to either augment existing culture results (for bacteria) or to detect and identify pathogens in the absence of culture (primarily for virus detection). The most widespread molecular methods are nucleic acid (NA) amplification techniques such as the polymerase chain reaction (PCR) (40). Advantages of PCR include high sensitivity that may detect very few microorganisms; availability of primer/probe sets for most common pathogens; routine extraction protocols for nucleic acid extraction and the development of automated systems and readouts for higher throughput of samples. Quantitative PCR can also provide quantitative data on the relative abundance of microorganisms that are present. Disadvantages include disassociation of the sample prevents microscopic evaluation of aggregated microorganisms; the detection sensitivity may not necessarily correspond to diagnostic sensitivity; potential sample contamination; complex samples containing inhibitors of PCR (such as eukaryotic DNA), and the potential amplification of DNA from nonviable microorganisms. Thus, PCR is a powerful approach that needs to be interpreted in the context of other diagnostic approaches and clinical data. FISH techonology is another sensitive and specific approach, which is particularly well suited

to the study of complex tissue samples and evaluation of the presence of microbial aggregates (41). FISH relies on hybridization of a fluorescently labeled probe to the 16S or 23S ribosomal RNA in bacteria or the 18S or 26S ribosomal subunits in eukaryotic microorganisms such as dimorphic fungal and protozoan pathogens. These molecular regions are specific to species level in microorganisms, and with careful optimization and use of controls, this approach can give robust *in situ* evidence of pathogens in a sample. Advantages of FISH include: culture-independent evidence of specific pathogens as spatially organized aggregates; *in situ* localization in the tissue and co-localization with other cell types (such as PMNs if used in conjunction with other RNA probes or stains); or other microbial members of a biofilm (such as in polymicrobial communities in dental biofilms), and demonstration of rRNA content specific to microorganisms indicating recent metabolic activity. Disadvantages of this approach include: this approach is expensive and time consuming and not useful for all diagnostic laboratories; the need for fixation and permeabilization of the sample; few commercially available probes for diagnostic use coupled with the need for testing and of validating new probes, and cost. Furthermore, FISH is not a stand-alone technique in the diagnostic setting, as culture is still used for antibiotic susceptibility testing. Immunohistochemical or immunofluorescent techniques represent another targeted approach to identifying pathogens in host tissue (42). Polyclonal or monoclonal antibodies specific to pathogens are routinely used to detect encapsulated pathogens in fluids such as *S. pneumoniae*, *Neisseria meningiditis*, and *Haemophilus influenzae*. These antibodies have not been consistently applied for the detection of bacteria in biofilms often because it is

thought the matrix may bind antibodies nonspecifically and there is a lack of commercially available antibodies specific for the many pathogens in the biofilm.

Treatment of biofilm infections is currently a difficult and complicated challenge. Generally, the strategies can be divided into involving a foreign body or not. In the infection not involving a foreign body, long-term treatment with high doses and often using combination of antibiotics with different killing mechanisms or physical-mechanical approaches (for example, high velocity spray and jet irrigators) that are aimed at biofilm disruption and removal; However, if a foreign body is involved, removal of the material is in most cases necessary for a successful outcome. In other cases, surface-coating or eluting substrates, which can be impregnated with antibiotics and/or antimicrobials (for example, acrylic beads with absorbable antibiotic-loaded bone cement to prevent orthopaedic infection) for biofilm prevention, where higher localized antibiotic concentrations can be achieved for longer periods by *in situ* antibiotic delivery compared to systemic administration (43). The strategy under development to target biofilm can be divided into three groups, (i) Targeting EPS by disrupting EPS synthesis and secretion or binding of EPS adhesins (44); targeting EPS chemical composition and structure (45); EPS-targeted antibodies and nucleic acid-binding proteins (46) ; (ii) inducing biofilm dispersal by regulating process that involves the degradation of the EPS matrix, and the triggering of this response has provided research strategies designed to promote biofilm self-disassembly, such as targeting cyclic-di-GMP pathway (47); targeting quorum sensing (48) and metabolic interference (49); and (iii) targeting dominant cells in biofilm, since available evidence shows that dormant cells or persisters residing within biofilms have a key role for drug tolerance. It is therefore attractive to consider

antimicrobial approaches that physically or chemically disrupt cells rather than interfering with cellular processes. Broad-spectrum cationic biguanides such as chlorhexidine or quaternary ammonium adhere to cell walls and disrupts cell membranes (50). However, penetration was limited over the expected timescales used in *ex-vivo* dental biofilms with longer term exposure increasing cytotoxicity, thus making this approach clinically impractical. Given the multifaceted nature of biofilm formation and the complex microbial interactions with the surrounding physical and chemical environment, a combination of multiple approaches may be required to successfully combat biofilm-mediated disease i.e., an efficient treatment of biofilm infections needs a well-established multidisciplinary collaboration, which include removal of the infected foreign bodies, selection of biofilm-active, sensitive and well-penetrating antibiotics, systemic or topical antibiotic administration in high dosage and combinations, administration of anti-quorum sensing or biofilm dispersal agents, phototherapy, targeting dormant cells and etc.

2.2. **Combinatorial drug library-based identification of specific biofilm bacterial ligands**

Theranostic agents have become the goal of biofilm diagnostic and treatment because of its specificity in attacking bacterial cells instead of normal tissues by focusing on trying to combine targeting molecules with diagnostic imaging agent and/or therapeutics into a single entity, thus enhancing their site-specific bacterial imaging and delivery while reducing off-targeting agents. More emphasis is now focused on identification of peptidomimetic molecules instead of monoclonal antibodies to be the better cell surface-targeting agents because they are (a) smaller and therefore might

penetrate biofilm better after conjugated with nanoparticles (24) ; (b) less likely to bind to the reticuloendothelial system such as liver, spleen, and bone marrow; and (c) easy to derivatize chemically (25). Therefore, peptidomimetic molecules will be the perfect match for the purpose to discover biofilm biomarkers for the development of the theranostic agent for biofilm infections. Combinatorial strategy has been proven to be a powerful tool for identification of synthetic ligands against specific biological targets (19-22). In this method, the OBOC library is prepared by a "split-mix synthesis" approach using polystyrene beads (88-micron diameter) as a solid support. As a result, each bead displays only one chemical entity and there are approximately 10^{13} copies of the same chemical entity (17). The schematic illustration of generation of OBOC combinatorial library is shown in Figure 1.1. This one-bead one-compound feature enables one to rapidly screen hundreds of thousands to millions of library beads with an on-bead binding or functional assays. The exact chemical nature of the compounds on selected beads with specific biological function can be determined with an automatic micro-sequencer. This technique has been applied to the discovery of targeting ligands against various biological targets, such as cancer cell surface receptor, protein kinase substrates and inhibitors, protease substrates and inhibitors, artificial enzymes, various ligands for the preparation of affinity column media and antibiotics (23-30).

2.3. Photodynamic therapy (PDT) and photothermal therapy (PTT)

Photosensitizer-enhanced phototherapy can be broadly classified into two categories; photodynamic therapy (PDT) and photothermal therapy (PTT). PDT requires three components: a light source, a photosensitizer (PS), and oxygen. When PS is irradiated by light of specific wavelength, it generates reactive oxygen species (ROS) that are

cytotoxic to the surrounding environment. Upon irradiation, the PS absorbs a photon and is transformed to an excited state from its ground state. Then it undergoes intersystem crossing to a triplet state accompanied by loss of energy as heat, fluorescence emission, or other photophysical energy. In the process of returning to the ground state, the PS creates reactive oxidizing species. In type I reactions, the PS directly reacts with substrates to produce radicals, whereas in type II reactions, molecular oxygen interacts with the triplet state PS to create reactive singlet oxygen. Type II reactions of photodynamic therapies are believed to be more relevant for PDT (13). Thus, targeted delivery of a PS to the intended area holds potential to pinpoint the site of therapeutic efficacy. PTT involves different mechanisms than PDT. Thermal homeostasis is crucial for optimal functioning of all biological activities. Contrast-enhanced PTT involves delivering a light-absorbing material to the region of interest and light-induced temperature elevation resulting in tissue ablation. Although the detailed interaction of the thermal effects on tissues and their surroundings are complex, nanomaterials-based PTT has been used to treat a variety of cancers *in vitro* and *in vivo* (51). PDT and PTT have risen as a minimally invasive antimicrobial approach for the treatment of localized infections in response to the problem of antimicrobial resistance (52). In theory, the use of photosensitizer to produce photothermal and photodynamical cytotoxic affect to bacteria has the following advantages: (I) Bacterial development of resistance to the PDT is less likely to happen as singlet oxygen and other free reactive species created by PDT interact with several cells structures and different metabolic pathway; (II) As PDT is non-invasive local therapy, following application of targeting sensitizer, a light source can be delivered into the target area precisely via a fiber optic cable, so the disturbances of the

microflora at other sites would not occur and the damage to the adjacent host tissues might be minimized if not avoided. (III) The PDT offers thorough irrigation elimination of pathogens in inaccessible areas of periodontal packet within short span of time, thus beneficial to both operator and the patients; (IV) The risk of bacteremia after periodontal debridement can be minimized; (V) There is no need to prescribe antibiotics, therefore the possibility of side effect and development of antibiotic resistant strains are avoided. (VI) Furthermore, the PDT and PTT can be applied repeated, and concern of toxic effect accumulation is less. However, hence far, the specificity of PS to accumulate in bacteria is poor, and thus clinical therapeutic results is limited. Therefore, a new targeting phototherapy utilizing high specificity against biofilm as well as increasing uptake of these pyrrole photosensitizers may highlight the ultimate translatable potential of PDT and PTT as a promising method to achieve successful disinfection, and infection treatments. The Schematic illustration of the mechanism of PDT and PTT was shown in Figure 1.4.

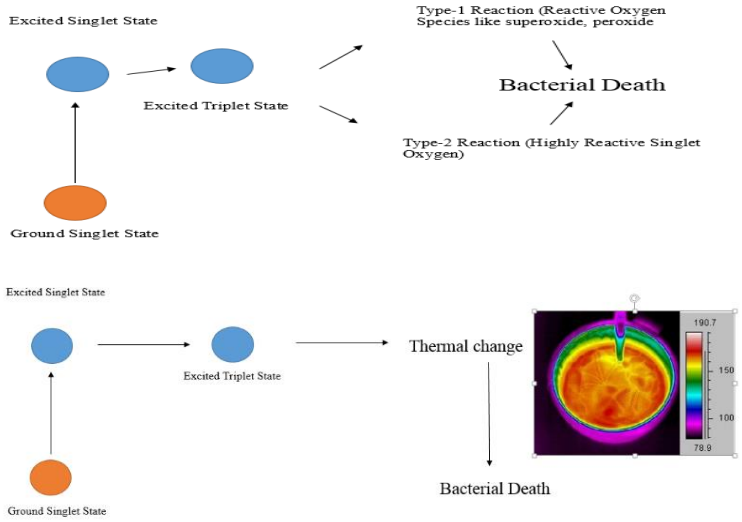


Figure 1.4. Schematic illustration of the mechanism of photodynamic and photothermal therapy.

2.4. Construction of nanocarriers with targeting phototherapy properties to inhibit resistant bacteria and biofilm infections

PDT is believed to be a potent method for biofilm treatments. However, undesired damage to normal cells may be caused due to the nonselective nature of PDT. Therefore, targeted PDT is preferred on one hand to enhance antimicrobial effects and one the other hand to reduce cytotoxicity to normal cells. However, considering limited diffusion distance of ROS (<50 nm) (53), accumulation of photosensitizers in or around bacteria is essential to conduct efficient antimicrobial PDT. NPs used for PTT and PDT possess many advantages such as high PS loading capacity and controlled release to increase the antibacterial properties. Passive targeting and active receptor targeting are two of the most commonly employed nanoparticle targeting methods. By modifying the size and surface characteristics, nanoparticles can be entrapped and accumulate in biostructures, commonly referred to as passive targeting. In comparison with passive targeting, active targeting stands out due to its potential for improved tissue targeting and accumulation usually via receptor mediated endocytosis. Based on the affinity of the ligands to specific receptors expressed on the surface of the cell, various ligands like antibodies, peptides, nucleic acids, proteins have been employed for active targeting for tumor studies. Examples of targeted nanomaterials for phototherapy on tumors include CRGD peptide, EFD peptide, Angiopep-2 peptide, transferring, folic acid and etc. (54, 55). So far, to our knowledge, there is no specific bacterial targeting nanoparticle based phototherapy combating biofilm reported.

Recently, the lab has developed several novel nano-carriers which possess dual-function for PDT and PTT. Using a reversible disulfide crosslinked micelle system named nano porphyrins (NPs), (PEG5k-Cys4-CA8 teloderdrimers), hydrophobic drugs can be encapsulated and triggered to be released at the tumor site and inside the cancer cells with high reductive potential. PEG5k-Cys4-CA8 loaded with PTX and decorated with ovarian cancer binding ligands (discovery through OBOC library screening), exhibit superior anti-tumor efficacy and lower systemic toxicity profile in dude mice bearing ovarian cancer tumor xenografts, when compared with equivalent doses to non-targeted PTX nanoparticles as well as clinical PTX formulation (Taxol R) (29-33).

In this project, we have employed OBOC combinatorial library technology to look for biofilm bacterial ligands for the improvement of screening and diagnostic techniques in parallel with the development of bacteria-targeted supramolecular photosensitizer delivery vehicles for photodynamic ablation against Biofilms.

CHAPTER 3. Identification of Broad-spectrum Biofilm Specific Ligands

3.1 Abstract: Background: The combinatorial strategy has been proven to be the powerful tool for the identification of synthetic ligands against specific biological targets. We have therefore employed combinatorial strategy to look for the specific biofilm bacterial binding ligands. **Material and method**: twenty-four combinatorial libraries were designed, synthesized, and screened against gram positive and gram-negative bacteria. After identifying suitable combinatorial libraries, that contain bacterial binding beads, the libraries were selected for the large-scale screening with bacteria and normal human cells. Only those compound-beads that bind strongly to bacteria and not bind to normal human keratinocytes, endothelial cells and granulocytes were selected and then placed in ABI automatic protein sequencer for Edman sequencing. The focused combinatorial library was then generated with bacterial binding motifs reserved to search for stronger broad-spectrum bacterial binding ligands. Briefly, the focused library was screened with multiple species of biofilm bacteria one by one. The compound beads with broad-spectrum bacterial binding, but without binding to human cells were finally isolated and the sequences and structures were determined by Edman sequencing. The bacterial binding compounds were then resynthesized in a large volume on Tentagel beads to test their binding ability and specificity. MTT assay was used to preliminary evaluate the potential cytotoxicity of these bacteria binding ligands. **Result**: four biofilm binding ligands were identified for their stronger binding properties to multiple bacteria, but not binding to normal human cells. Therefore, they were selected as lead compounds for the development of biofilm imaging probes. **Conclusion**: four specific biofilm binding ligands

were identify by using OBOC combinatorial library strategy. *In vitro* test demonstrated that they posse the broad-spectrum bacterial binding ability, but not bind to normal human cells. Initial MTT assay indicated that four ligands do not have cytotoxicity to human endothelial cells. Zonal inhibition assay illustrated that four bacterial binding ligands are not antibiotics.

3.2 Introduction

Biofilms are notoriously difficult to diagnose and treatment. Current means of diagnosis of biofilm associated infection (BAI) are lacking [23-25]. The diagnostic tools that can determine whether or not the patient is suffering from a *BAI* infection as well as one that localizes at the infection location would provide increased sensitivity and specificity. Therefore, highly sensitive and specific biomarkers for bacterial biofilm are urgently needed. Biofilm bacteria biomarkers can be any single molecule or a combination of more than one cellular molecule including DNA, RNA, proteins, peptides, carbohydrates, or small metabolites that are disease-specific and could be measured in order to analyze and monitor disease progression [34,35,37]. Peptides and peptidomimetics are alternative classes of targeting agents that can be used to target bacterial cells, with several advantages over antibodies. These include less non-specific binding to the reticuloendothelial system (mononuclear phagocyte system which consists of phagocytic cells, mainly monocytes and macrophages), and ease of conjugation to therapeutic payload and less expensive to manufacture. Peptides are normally susceptible to proteolytic degradation. However, with the use of D-amino acids, unnatural amino acids and organic moieties, proteolytic stable peptides and peptidomimetics can be developed for targeting cell surface proteins of cancer cells. In this study, we employed OBOC

combinatorial library approach to identify biofilm bacteria specific peptides as the biomarkers.

3.3. Material and methods

3.3.1. Cells used for the studies. *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *E. coli*, *E. Faecalis*, *K. Pneumonia*, *C. amalonaticus*, *P. gingivalis* were purchased from ATCC. They are not antibiotic resistant strains. HaCat: (site: skin), Huvec (human endothelial cells), oral epithelial cells and NUFF cells (new born human foreskin fibroblast cells) were purchased from ATCC. Normal human granulocytes were obtained from the healthy volunteer. Rink amide MBHA resin (0.5 mmol/g), Fmoc-protected amino acids, and N-hydroxybenzotriazole (HOBt) were purchased from GL Biochem (shanghai, China). 1-3-Diisopropylcarbodiimide (DIC) was purchased from Advanced Chem Tech (Louisville, KY, USA). TentaGel S NH_2 resin (0.24 mmol/g, 1% DVB cross-linked 90 um) was purchased from Rapp Polymere (Tubingen, Germany). The other chemical reagents were purchased from Aldrich (Milwaukee, WI, USA).

3.3.2 Generation of random and focused OBOC libraries. Twenty-four random and focused small molecule libraries were generated by using a split-mix synthesis approach as previously described (17). Standard solid phase peptide synthesis method with 9-Fluorenylmethoxycarbonyl (Fmoc) chemistry was used to synthesize the OBOC libraries. Briefly, the peptide was synthesized on 88 μm diameter Tentagel beads (polyethylene glycol-grafted beads resin, with loading 0.25 mmol/g, from Rapp Polymere), using 1-hydroxybenzotriazole/1, 3-diisopropylcarbodiimide as coupling reagents. Three-fold molar excess of Fmoc-protected amino acids to resin was used for coupling. Completion

of coupling and Fmoc deprotection was monitored by the ninhydrin test. The disulfide formation was achieved with gentle mixing of the bead-library in 20% DMSO in ammonia acetate buffer (pH 6.2). These libraries contained tens of millions compound beads with each carrying a peptide with distinct natural and unnatural amino acid or small organic molecule moieties. The twenty-four combinatorial libraries used in these studies, including X1 focused combinatorial library, RGD based combinatorial library, seven cyclic combinatorial library and fifteen random combinatorial libraries.

3.3.3. Small-scale screening. Biofilms were created by inoculating *E.coli,* S. aureus and *S. mutants* into tissue flask separately under shaking at 60 rpm at37C. After bacteria attached to the surface of tissue flask, unattached bacteria were removed; and fresh media was replaced every three days to allow biofilm formation. After two weeks, tissue flasks were washed to remove the free bacteria, the 14-day biofilm bacteria were scraped with a sterile blade. The scraped off bacteria were placed in PBS and ultra-sonicated at a low speed in a water bath ultrasonic cleaner for 10 mins to disturbed the clumps. The biofilm bacteria were then labeled with live gram staining kit (molecular probe). The biofilm bacteria were then screened with compound libraries (~15,000 beads) to identify possible compound library which may have bacterial binding beads for large scale screening.

3.3.4. Large-scale screening assay. The OBOC combinatorial libraries selected from small-scale screening were screened in large scale (~750,000 beads/each library) using biofilm *E. coli*, salivary bacteria *S.mutans* and *S. aureus* to look for compound beads that bind to all three bacteria. In brief, biofilm *E.coli* were screened with OBOC libraries for 1 hour. Beads with unique binding ligands that interacted with cell surface receptors were coated by one or more layers of the *E. coli* cells. These positive beads were isolated

and the binding cells were stripped off by using 8M guanidine hydrochloride and then screened with *S.mutans* and *S.aurues*. The compound beads which bind to all three bacteria were sorted.

3.3.5 Specificity filtering assay. Specificity filtering assay was employed to identify the ligands which only bind to bacteria, but not to normal human cells. Briefly, the biofilm bacterial binding beads identified from the previous large-scale screening were incubated with live human keratinocytes (HaCat), endothelial cells (Huvec), fibroblast cells (NUFF), oral epithelial cells and freshly isolated granulocytes separately. Those compound beads with strong binding to bacterial cells but not bind to normal human cells were finally selected. The chemical structures of selected beads were determined by Edman chemistry using an automatic ABI-protein sequencer.

3.3.6 Focused library screen assay for broad spectrum biofilm ligand identification. The focused compound library was generated with a chemical motif reserved and biased on double layer beads, where only 10% of surface of beads were functional, while 90 % inside of beads were block for the purpose of decoding. The focused library was screened with multiple bacteria (*E. Faecalis*, *K. Pneumonia*, *C. amalonaticus*, .*Citrobater* sp, *Bacillus* sp, *E.coli*, S.*mutans*, *P.aeruginosa* and *P. gingivalis*) one by one using method described above. The beads which have all bacterial binding to different strains were sorted and incubated with human cells, including HaCat, HUVEC, epithelial cell and granulocytes. The beads with bacterial binding, but not binding to normal human cells were finally identified. The sequencings and structures of identified compounds were determined by Edman chemistry.

3.3.7 Cytotoxicity evaluation assays. Those compound beads with high binding affinity and specificity for biofilm bacteria were resynthesized in soluble aqueous form. MTT assay was used to evaluate the potential cytotoxicity of the biofilm compounds identified on normal human keratinocytes (56). Briefly, human keratinocytes were cultured on 96-well plate overnight. The serially diluted ligands were added to each well and incubated for 4 hours before adding MTT (3-[4,5-dimethyl-thiazol-z-yl] 2,5 diphenyl tetrazolium bromide) solution (5μg/ml in PBS). After 4 hours of incubation, DMSO-containing solution was added. The optical density at 540 nm is measured with auto-reader.

3.3.8. Zonal inhibition assay to evaluate compounds' bactericidal property

Zonal inhibition test was also used to evaluate the potential toxicity of identified compounds to bacteria. Briefly, *E.coli* and *S. aureus* were grown in pure culture. 10 ul of suspension of pure culture is spread evenly over the face of a sterile agar plate. 20, 40, 60 80 ug/ml of tested compounds were applied to the agar plate and incubated for 48 hours. The size of zone inhibition was examined. Antibiotics Cipro was used as the control.

3.4. Results

3.4.1 Creating biofilm on tissue flasks to obtain biofilm bacteria. *E.coli*, S. *aureus* and S. *mutans* were inoculated into tissue flasks. After cell attached to the surface, free bacteria were removed, and fresh media was replaced every three days. As shown in figure 3.1, bacterial biofilm were created (a) *E.coli*, (b) *S.aurues* and (c) *S.mutans*. These biofilm cells were removed from tissue flasks and used the screening with OBOC libraries.

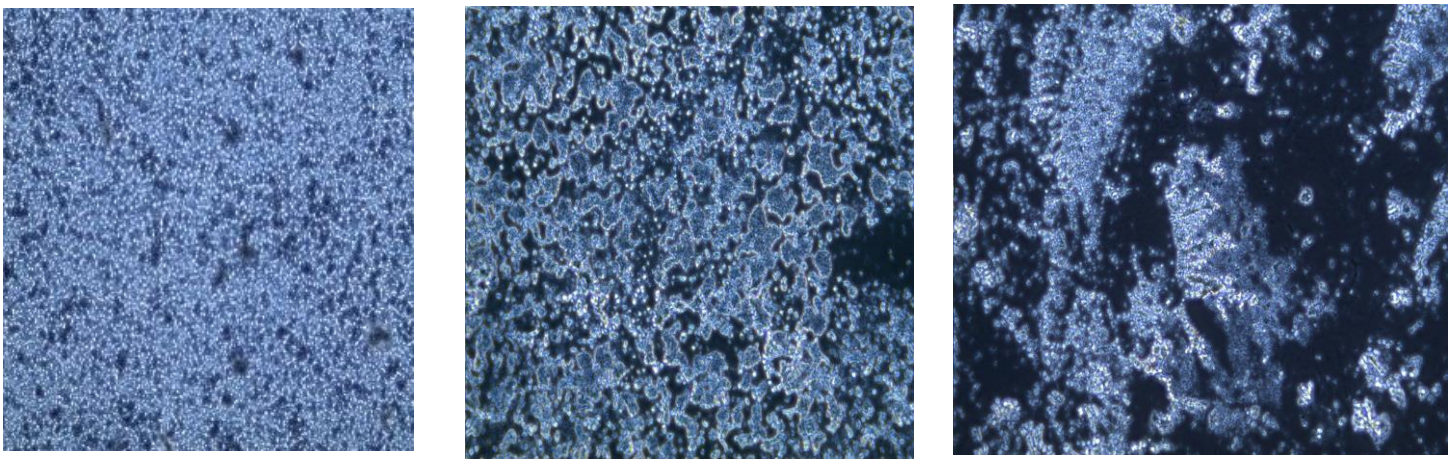


Figure 3.1 . Bacterial biofilm were created (a) *E.coli*, (b) *S.aurues* and (c) *S.mutans*. These biofilm cells were removed from tissue flasks and used for the screen.

3.4.2 Identification of bacterial binding beads from using intact live cell assay.

Biofilm bacteria were labeled with live gram staining kit and screened with 24 libraries in small scale (about 15,000 compound beads at each screening). Six libraries were selected for the large-scale screening (about 750000 beads), including X1 focused library, RGD based library, and four linear libraries. As

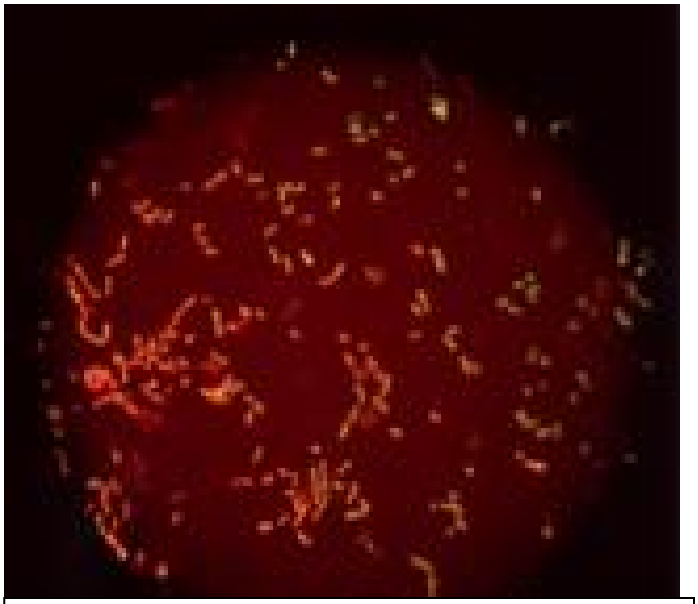


Figure 3.2.. Compound bead with bacterial binding; bacilli shape (*E.coli*); chain (*S.mutans*) and cocci (*S.aureus*).

shown in Figure 3.2, the compound bead with *E.coli, S.mutans* and *S. aureus* binding were identified.

3.4.3 specificity filtering assay: to identify non-human keratinocyte binding beads from the bacterial binding beads selected from 3.4.2. Human endothelial cells were grown in Epi-Life media. After harvested by trypsin, keratinocytes cells were screened with bacteria binding beads sorted from 3.4.2 for 4 hours in 5% CO_2 shaker at 50 rpm. As shown in Figure 3.3, some bacteria binding beads has more Hacat cell binding, some has less HaCat cell binding, and one non-binding beads was identified from bacterial binding beads (arrow). All of the HaCat cell non-binding beads selected from the study were then rescreened with human epithelial cells, and again the negative beads which have no endothelial cell binding were selected and so on. Among the six OBOC libraries screened, only the linear OBOC library showed specificity binding to bacteria but not bind to HaCat, endothelial cells and granulocytes. A total of 321 bacteria binding beads were identified from a linear OBOC library, and 13 compound beads were finally identified with the properties of binding to bacteria but not to normal human cells. The chemical structures of 13 peptides were determined by

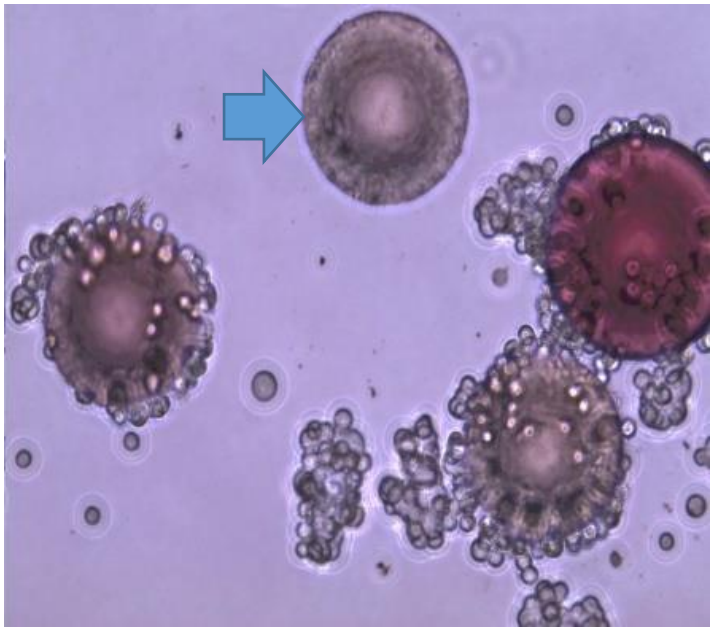


Figure 3.3. Micrograph of bacterial binding beads screened with human HaCat cells. One bacterial bead has no human cell binding (arrow).

Edman sequencing. The 13 peptides were resynthesized and challenged with *E.coli*, *S. aureus* and *S. mutans* again. One compound with all bacterial binding ability and also no net charge was elected as the lead compound to generated focused OBOC library.

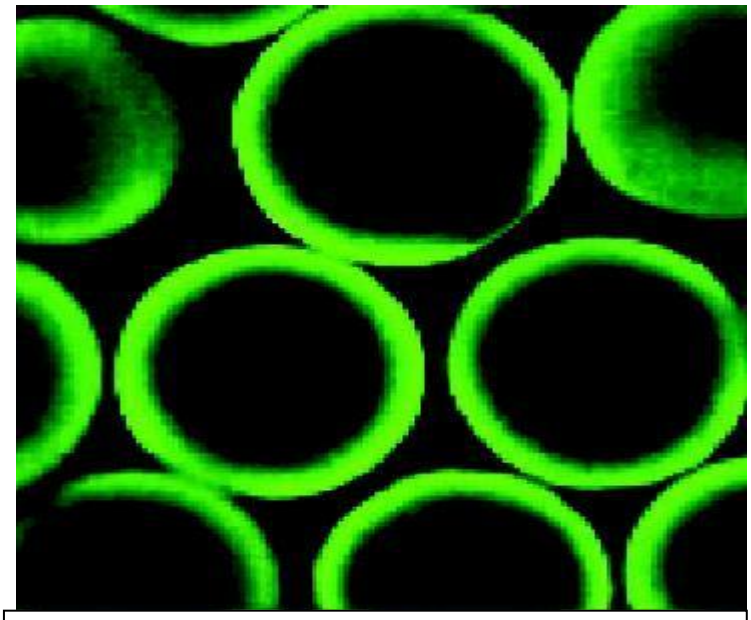


Figure 3.4. Confocal image of double layer beads used to generate focused OBOC library. Fluorescent sections are functionalized layer for synthesis of compounds.

3.4.4. Focused OBOC library screening to search for broad-spectrum biofilm ligands. Bacterial binding motif based focused OBOC library was generated with down substitution, ie only 10% surface of beads are functional, while 90% inside of inside of beads were blocked as the coding tag. Figure 3.4 shows the double layers beads with only 10% functional layer (florescent) used to generate focused library. In this way, compound density on the beads

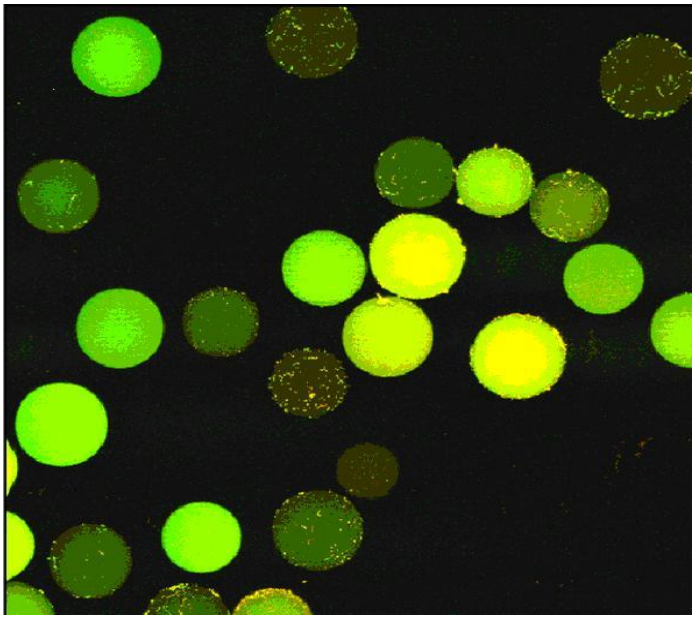


Figure 3.5. Confocal image of focused OBOC library screened with *E.coli,* showing much more bacterial binding beads than initial OBOC library.

will be reduced thus helping to find stronger binding ligands. As shown in Figure 3.5, there were much more bacterial binding beads in focused OBOC library than initial ones. The focused OBOC library was then screened with multiple species of bacteria one by one, including *S. aureus, S. epidermidis, P. aeruginosa, E.coli, E. faecalis, K.pneumonia, C.amlonaisus, S.mutans and P. gingivalis* one by one. Briefly, biofilm bacterial *E.coli* were incubated with focused library and the strong binding beads were sorted (Figure 3.6). The *E.coli* binding beads were treated with 8M Guanidine to get rid of the binding bacteria and then sterilized with 70% alcohol, then enter to the next cycle of screening with different bacteria

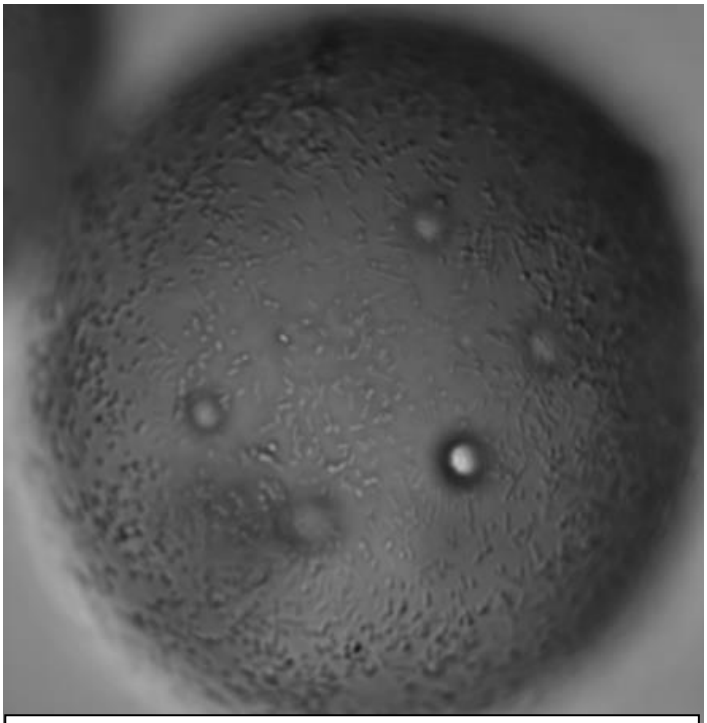


Figure 3.6. SEM image of focused OBOC library screened with *E.coli.* .Whole bead was covered bv bacteria after one hour incubation.

bacterial binding beads than initial OBOC libraries. The compound beads with all of above bacteria binding were then screened with normal human cells, including endothelial cells (Huvec), NHK, fibroblast cell, epithelial cells and granulocyte (Figure 3.7). Thirteen compound beads with bacterial binding, but no binding to human cells were

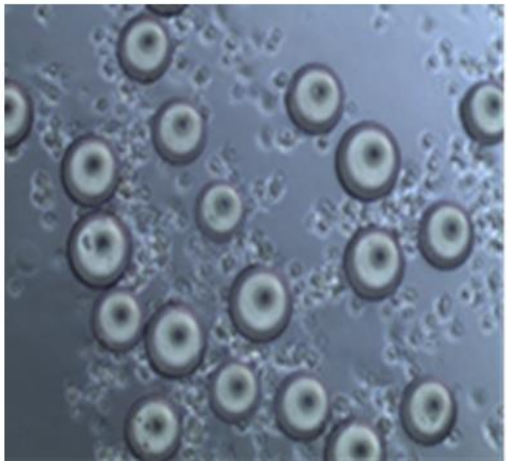


Figure 3.7. The bacteria binding compounds were then screened with

finally identified, and their structure and sequences were determined by Edman chemistry.

3.4.5. Evaluation of biofilm bacteria ligands' binding ability and specificity using hit validation assay. Thirteen bacteria binding compounds (named Bio1-13) were re-synthesized on Tentagel beads according to their structure and sequences. Biofilm bacteria were incubated with each of the 13 compoun-beads for 1 hour in saline. As shown in Figure 3.8, the Bio5 compound beads with mix bacteria binding, including *S.aureus and E.faecalis* (a), *S.mutans and P.aeruginosa* (b), *K. pneumonia and C. amlonaisus* (c) and *P.gingivitis* (d); while there were no bacterial binding on the control beads which have no compounds on the surfaces. Figure 3.9 is the SEM image of *P.aeruginosa* and *S.aureus* bind to the Bio5 compound bead. Both SEM and Confocal images have shown that Bio4 was able to bind to mixed bacteria biofilm.

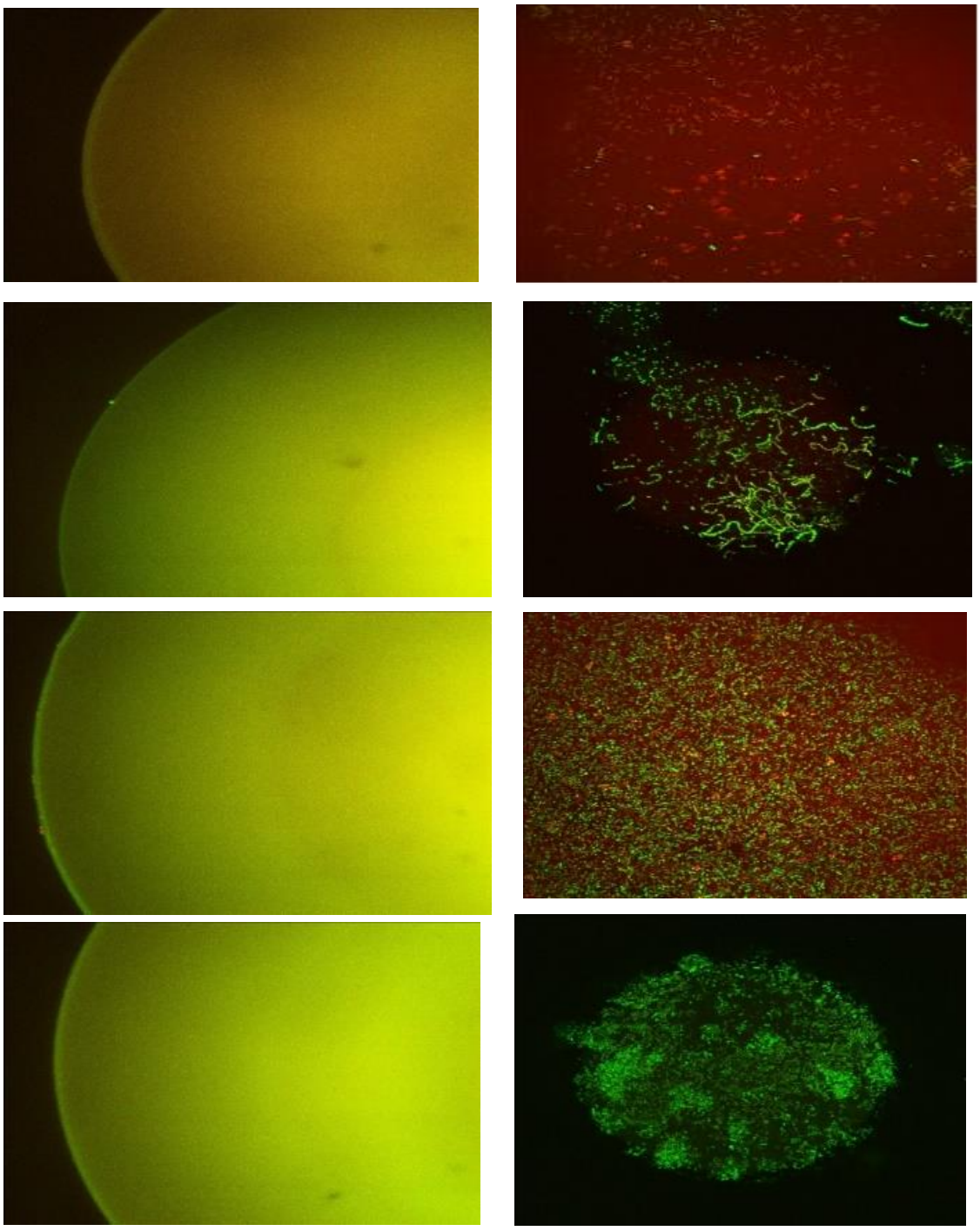


Figure 3.8. Confocal images of mix bacterial binding to compound beads, including *S.aureus and E.faecalis* (a), *S.mutans and P.aeruginosa* (b), *K. pneumonia and C. amlonaisus* (c) and *P.gingivitis* (d Left images are are the control beads without compounds.

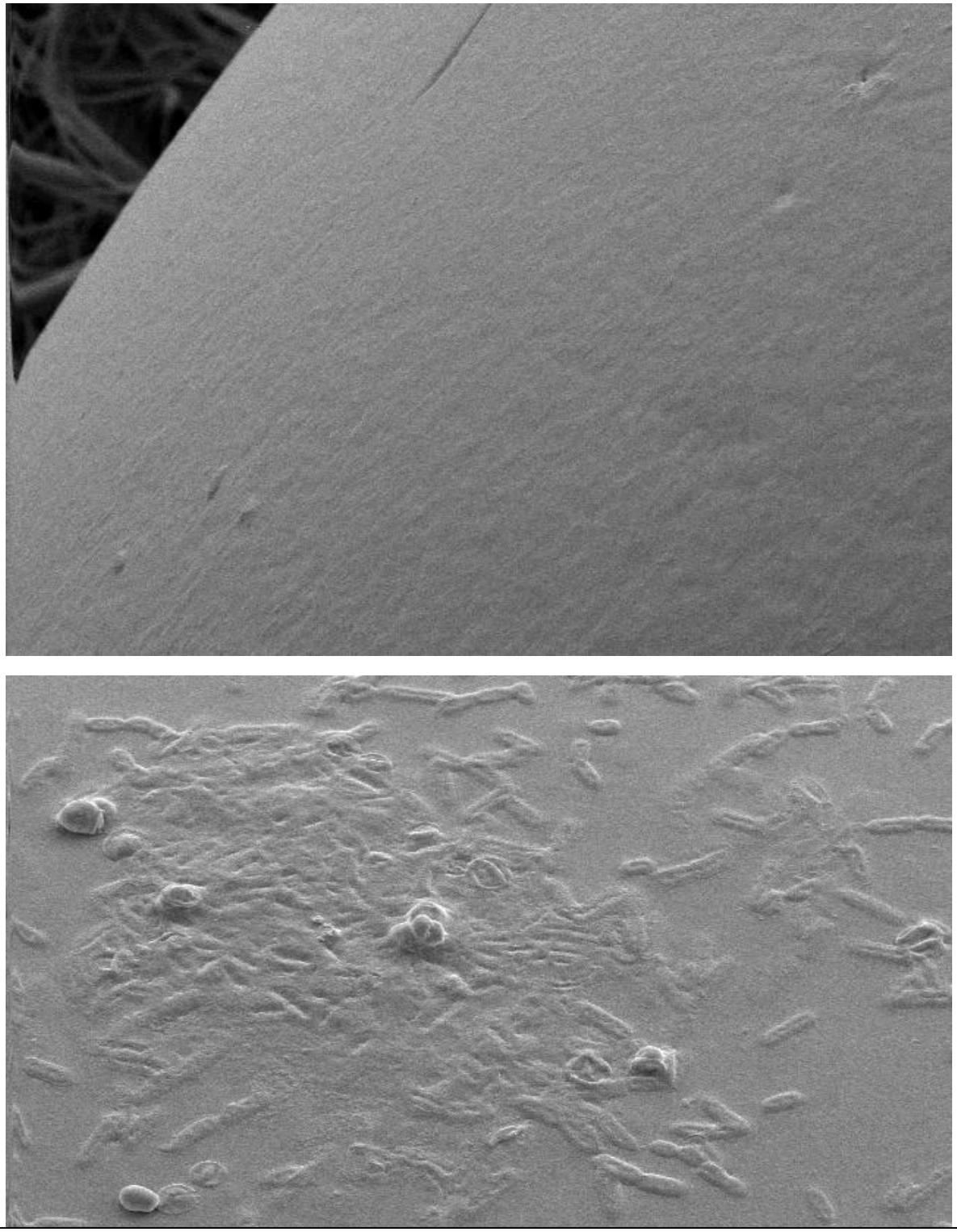


Figure 3.9: SEM image of *P.aeruginosa* and *S.aureus* binding to the Bio5 compound bead, whe bacilli shaped are *P.aeruginosa;* cocci are *S. aureus (*bottom*)* (control bead (top) without bacte binding.

3.4.6 Potential cytotoxicity evaluation of identified compounds from MTT essay. Six bacteria binding ligands selected from the focused library against broad spectrum of bacteria were synthesized in liquid form. The ligands were purified by HPLC and the molecular weights were confirmed by MS. The MTT assay indicated there was no obvious

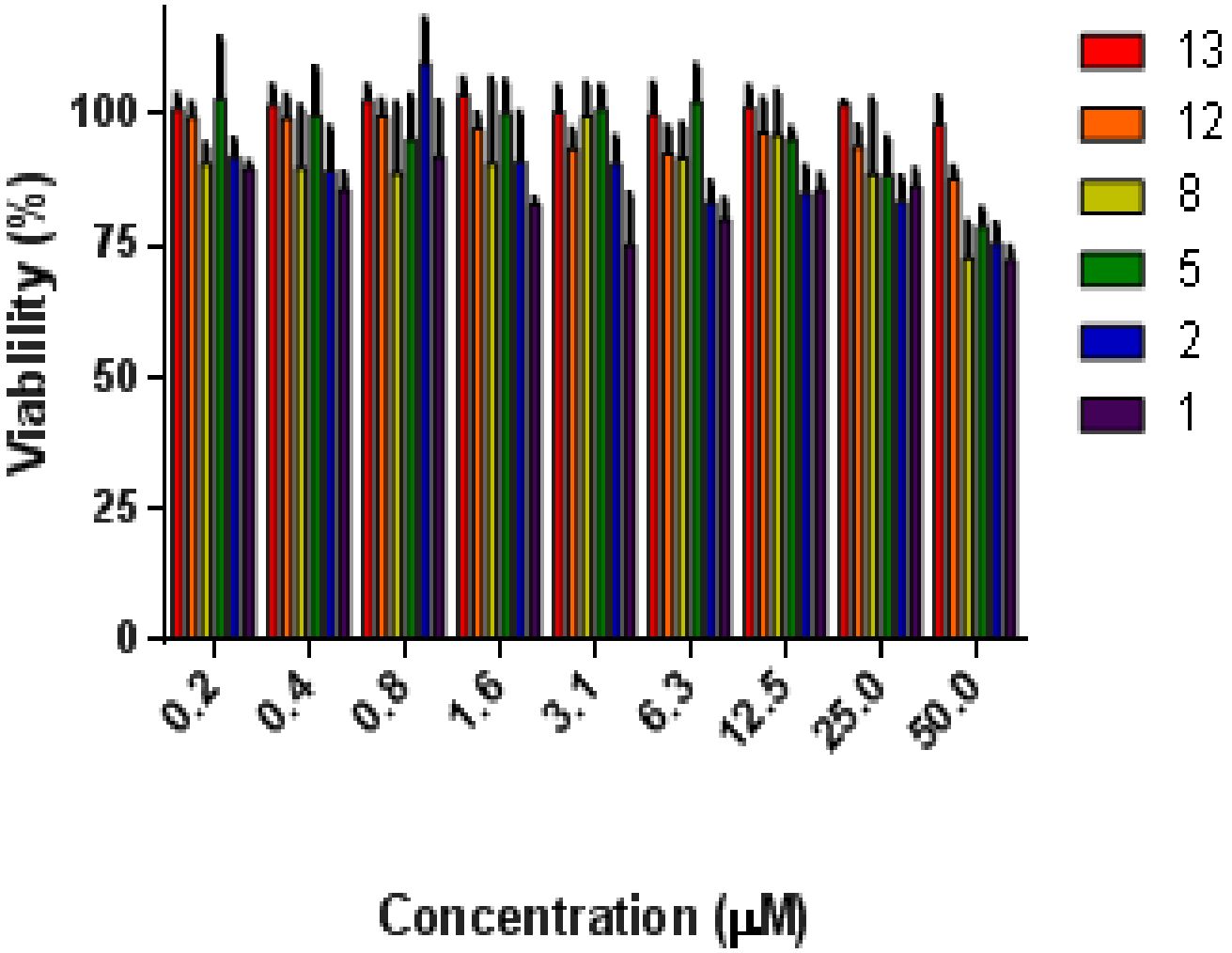


Figure 3.10. MTT results of six identified bacterial compounds on human Hacat cells. There are no Cytotoxicity on four ligands; compounds 1 and 2 are questionable, thus ruling out form experiment.

cytotoxicity observed on four compounds up to 50 um (Figure 3.10), while Bio 1 and 2 are questionably and rule out from the experiments.

Zonal inhibition assay was used to evaluate whether bacterial binding compounds possess bactericidal effect. As shown in Figure 3.11, no inhibition zone showed on the Bio 5 compound on the zonal incubation plates; while there were inhibition zones shown up on

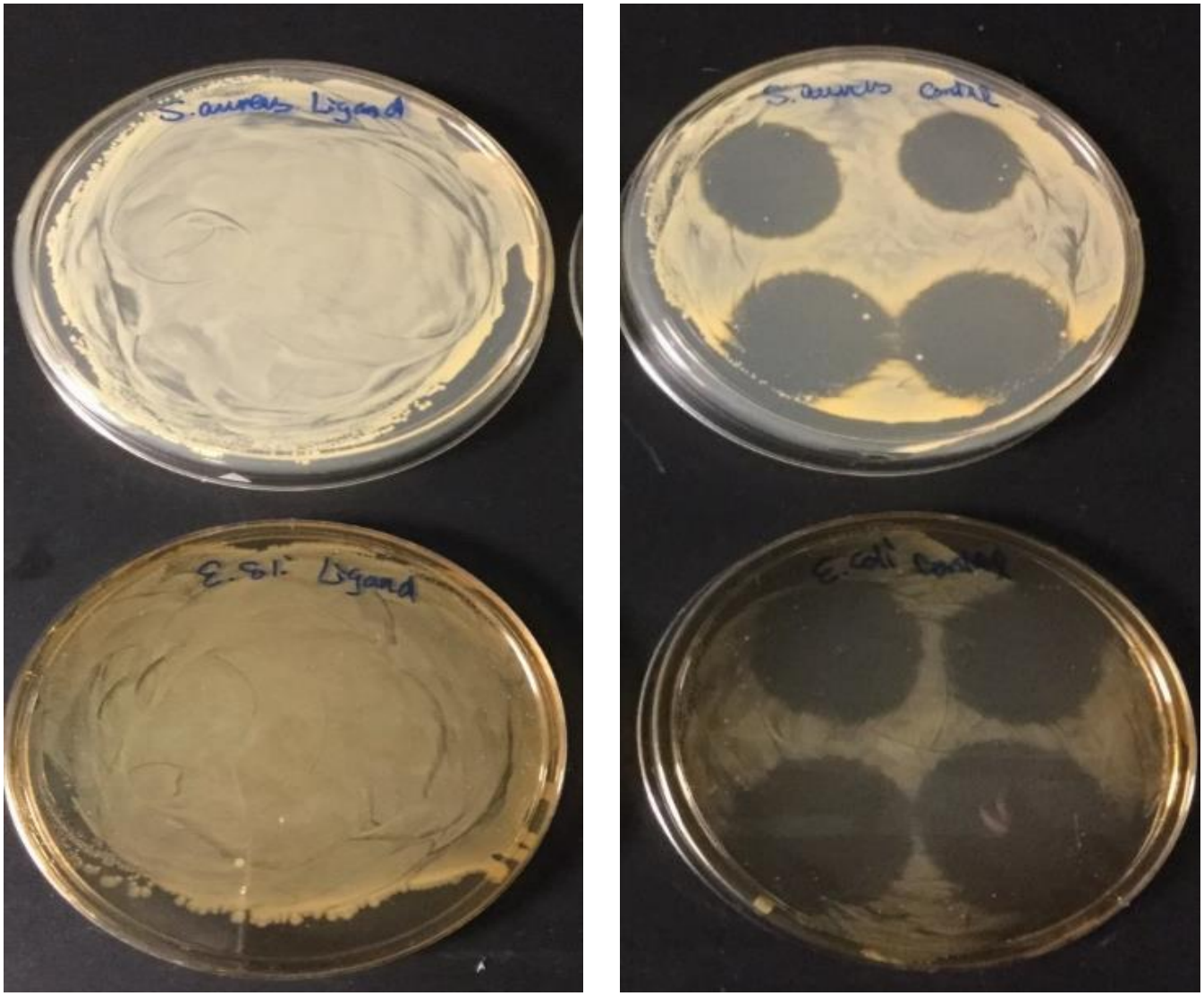


Figure 3.11. Photograph of zonal inhibition results of tested compounds. There is no inhibition zone shown on the tested Bio5 (left), while there are inhibition zones shown on the control groups (right).

the Cipro plates. Therefore, the bacterial binding compound identified here have no bactericidal effect.

3.4. Discussion

Focused combinatorial library makes it available to obtain large volume of compound beads for many concurrent ligand screenings. Only strong binding beads with full coverage of bacteria were selected for the next round of screening. Among the several approaches to search for the biomarkers, the biological library (such as phage-display library) and combinatorial library are the two most commonly used methods to identify cancer-targeting peptide ligands (57). OBOC combinatorial library method was developed over two decades ago by my mentor (17). Thousands to millions of compound beads can be generated and scanned rapidly. Each compound bead in OBOC combinatorial library displays only one chemical entity. One major advantage of the OBOC method over phage-display method is that D-amino acids, unnatural amino acids, and organic moieties can be easily incorporated into the construction of the OBOC libraries, rending the identified ligands containing such building blocks more resistant to proteolysis, while biological combinatorial libraries are generally limited to L-amino acids,

Among the six libraries selected for the large-scale screening studies, a total about 4,500,000 compound beads were screened, we identified 546 biofilm bacteria binding beads. Following the specificity filtering assay, only thirteen compound-beads from 137 positive compound beads were identified from linear focused library. Although we identified a lot of bacterial binding compound beads from other cyclic libraries, however,

these beads also bind to human cells, making their binding not bacteria specific. One of advantages of synthesizing focused OBOC library is that the focused OBOC library make it available to obtain quite a large amount of bacterial binding beads with increased chemical permutations, therefore allowing multiple screening cycle with different libraries, until the ideal compound beads were identified.

A significant challenge facing OBOC screening approaches is to have a robust method to reduce or eliminate false negative compound-beads when screen with normal human cells. This issue needs to be overcome by keeping normal human cells in a healthy and proliferative status. To maintain this screening situation, we usually balanced the incubation media overnight in the incubator with 5% CO_2; when we performed the screening assay, the shaker was equipped with 5% CO_2. After trypsinization, the human cells were usually incubated in the 37°C shaker with CO_2 for one hour to help resume the cell in healthy status before adding the OBOC library beads.

During the multiple bacteria screening cycles, one of the technical difficulties is not to lose the identified compound-beads during the multiple screening steps, as the beads are small (~88μm) and not easily visible to naked eye. For example, after one putative positive bead was identified, it has to be treated with 8M guanidine to strip of the binding cells and then sterilized with 70% alcohol; washes with dd H_2O and PBS three times each before moving to the next screening. Therefore, it is not uncommon to lose important identified beads during the washed and treating steps. To overcome this issue, the entire bacteria binding beads were transferred to the 1.5 ml centrifuged tube and then 8 M guanidine was added and incubated for 10 minutes to get rid of the binding bacteria. 1 ml of DD water was added to the centrifuged tube and spun for 3 minutes at 1200 rpm.

The beads selected were then washed for 3 x in DD water, 1x of 70 % alcohol and 3 x PBS.

3.5. Conclusion

Four broad-spectrum bacterial ligands were identified for possessing the property of specifically binding to bacteria, *including S.aureus, E.faecalis*, *S.mutans ,P.aeruginosa* , *K. pneumonia , C. amlonaisus*, *P.gingivitis* but not binding to normal human oral cells. MTT assays indicated that these four ligands have no cytotoxicity to human endothelial cells at up to 50 uM. Zonal inhibition assay indicated these four ligands do not have bactericidal effects; therefore, they are not antibiotics. These four bacterial ligands were selected for the development of biofilm imagine probes.

CHAPTER 4. Development of biofilm probes and evaluation of their targeting efficacy

4.1. Abstract

Background: Reliance on culturing as the 'gold standard' of medical microbiology exclusively for the identification of bacterial pathogens as a diagnostic criterion in clinical laboratories is not clear-cut with BAI. The development of molecular-based diagnostic approaches to BAI is central to improving the detection and identification of microorganisms and establishing their role in pathogenesis. Through screening twenty-four combinatorial libraries against multiple species of bacteria, four peptide ligands were identified with the broad-spectrum biofilm bacteria binding properties, including *S.aureus, E.faecalis*, *S.mutans ,P.aeruginosa* , *K. pneumonia , C. amlonaisus*, *P.gingivitis* , but not binding to human keratinocytes, endothelial cells and WBC, therefore, these four putative bacterial ligands were selected for further development of the biofilm optical probes. **Material and methods:** The optical bacteria imaging probes were generated by biotinylating the ligands at the carboxyl end via hydrophilic spacer to minimize the interference by streptavidin. Peptide-histochemistry assay was employed *in vitro* to evaluate these biofilm probes for targeting efficacy and specificity to multiple species of biofilm grown on the chamber slides through streptavidin 488. **Results**: Two biofilm probes, Bio5 and Bio12 were able to stain multispecies biofilm grown on the chamber slides at the concentration of 10 μM after 30 min incubation. **Conclusion**: biofilm probe 5 and 12 were able to detect multispecies biofilm through streptavidin. Therefore, they are selected to construct bacteria-targeted

supramolecular photosensitizer delivery vehicles for photodynamic ablation against biofilm.

4.2. Introduction

It has been recognized that microbial biofilms are responsible for up to 80% of infections in humans. They can cause diverse disease to human bodies, including dental caries, periodontitis, endodonititis, cystic fibrosis, otitis media, pneumonia, and osteomyelitis and so on. Recently dental implants have been a focus of study for oral biofilms that may eventually lead to peri-implantitis with loss of the supporting bone and ultimately failure of the implant. Currently there are several methods which have been used by clinical microbiologist for detection and measurement of microbial biofilm in response to agents (table 1-2) (57,58,).

Method	Action of application	Aim
Roll plate	Extraluminal biofilm detection	Growth of biofilm-embedded bacteria
Sonication, vortex, and plate counting	Intraluminal and extraluminal biofilm detection	Growth of biofilm-embedded bacteria
Acridine orange staining	Extraluminal biofilm detection	Direct investigation of biofilm produced on catheter by microscopy
Streak plating of alginate swab	Investigation of biofilm produced on indwelling catheter	Growth of biofilm-embedded bacteria

Table 1. The methods used for detection and measurement of biofilms produced on medical devices.

Method	Aim
Tube method (TM)	Qualitative detection by observing biofilm lined on bottom and walls of tube
Congo red agar (CRA)	Qualitative detection by observing colony color change
Microtiter plate (MtP)	Quantitative detection of biofilm by microplate reader (microELISA)
Real-time PCR	Detection of biofilm genes
Fish	Detection of bacterial RNA

Table 2. The methods used for detection of biofilm

However, reliance on culture as the 'gold standard' of medical microbiology exclusively for the identification of bacterial pathogens as a diagnostic criterion in clinical laboratories is not clear-cut with BAI. Numerous publications indicate a discrepancy between culture and molecular diagnostic methods. Thus, it is a huge challenge to develop potential diagnostic and the therapeutic strategies against microbial biofilms. In the previous aim, through screening focused combinatorial library, we identified four biofilm peptides with the property of specific binding to biofilm bacteria on beads. In this aim, we will develop biofilm imaging optical probe by using these peptides to assess their biofilm targeting efficacy for the purpose of development of theranostic agent to combat biofilm.

4.3. Methodology

4.3.1. Development of biofilm optical probes using the four peptides discovered from screening combinatorial libraries.

Materials: Rink amide MBHA resin (0.5 mmol/g), Fmoc-protected amino acids, and N-hydroxybenzotriazole (HOBt) were purchased from GL Biochem (shanghai, China). 1-3-Diisopropylcarbodiimide (DIC) was purchased from Advanced Chem Tech (Louisville, KY, USA). TentaGel S NH_2 resin (0.24 mmol/g, 1% DVB cross-linked 90 um) was purchased from Rapp Polymere (Tubingen, Germany). The other chemical reagents were purchased from Aldrich (Milwaukee, WI, USA) and were of analytical grade.

Generation of biofilm probes using the four peptides discovered**.** The schematic illustration of generation of biofim probes is shown in Figure 4.1. Briefly, biofilm probes, named as Bio 5,Bio 8, Bio 12, and Bio 13 were synthesized on Rink resin (loading 0.5 mmol/g) using the above described Fmoc chemistry. Peptide-linker-lysine (biotin) was prepared on Rink resin using Fmoc-Lys(Alloc) as the first building block. After Alloc deportation, biotin was coupled to amino group at the ε position of lysine. Fmoc-Ebes linker and Fmoc amino acids were coupled to resin sequentially, followed by Fmoc deportation. The probes were finally cleaved from the Rink resins using the TFA cocktail and then precipitated in cold ether. Disulfide peptide cyclization was carried out using CLEAR-OX resin (Peptides Intl. Louisville, KY, USA). OSCC probes were purified by preparative reverse-phase (C18) high-performance liquid chromatography (RP-HPCL). Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) was employed to verify the final biotinylated biofilm probes.

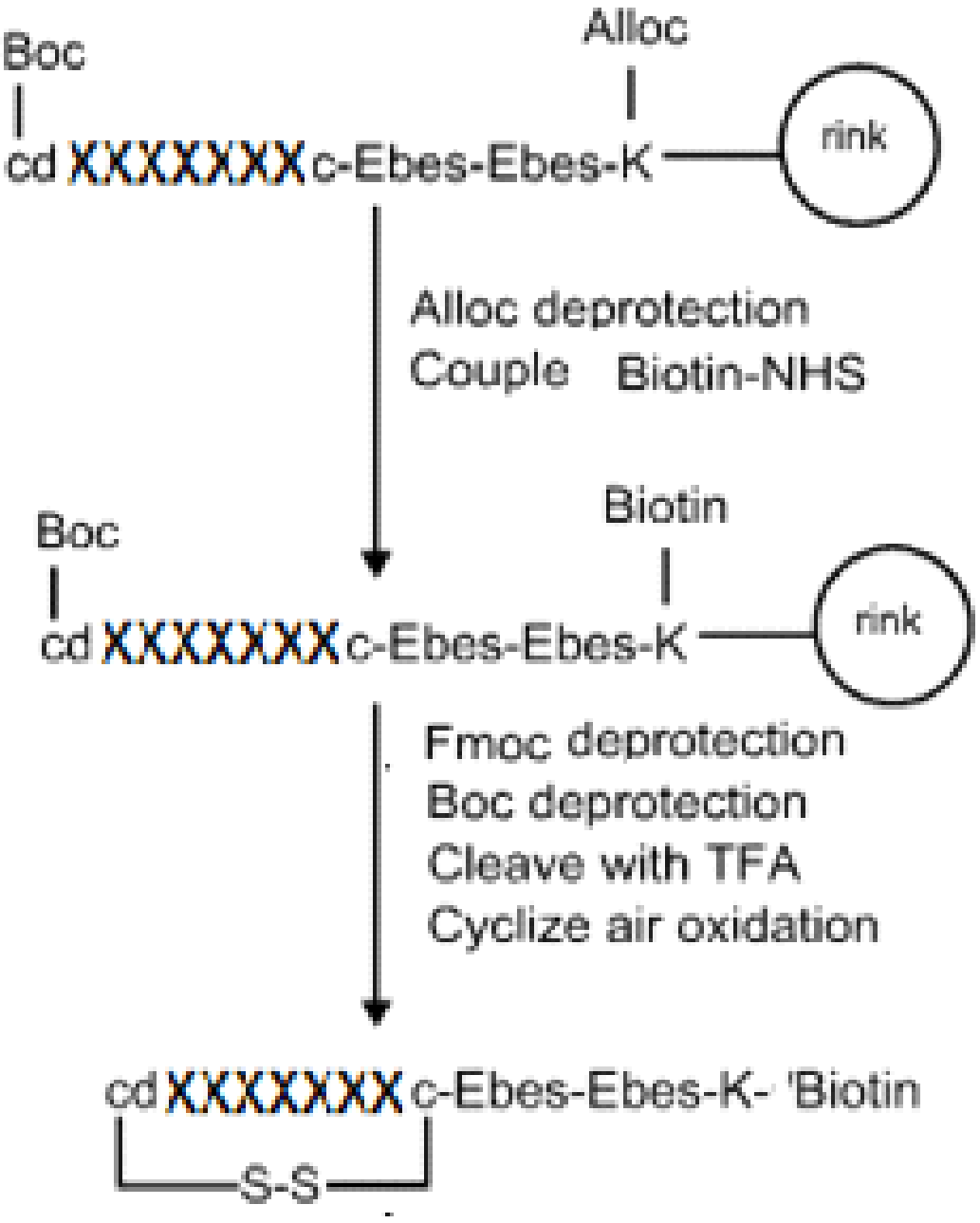


Figure 4.1: Flowchart of demonstrating of generation of biofilm probes

4.3.2. Peptide-histochemistry studies to evaluate binding efficacy and specificity of optical probes on biofilm (58). Optical biofilm probes generated from 4.3.1 were used for the studies. Multiple species of biofilm was created by inoculated *E. coli*, *S. mutans* and *S. aureus* into chamber slides and incubated at 37º C at 50 RPM overnight. After bacteria adherent to the surface of chamber slides, free bacteria were removed and fresh culture media BIH was replaced every three days to promote biofilm growth. After two weeks, 14-day multispecies biofilm were incubated with 5% BSA to block non-specific binding. After washing with PBS 5X, biofilm probes of different concentrations were added to the biofilm grown in the chamber slides and incubated for half an hour, then washed 3X with PBS, followed by adding 1:500 dilution of streptavidin-Alexa 488 and incubation for half hour. Specimens were washed 3X with PBS and fixed briefly with 4% formaldehyde before adding the DAPI. Confocal microscopy was then performed.

4.4. Results

Although four peptides bind to bacteria strongly on the solid support of polystyrene beads, we need to evaluate their bacterial targeting ability in solution form. Therefore, peptides were synthesized in solution form to generate biotinylated optical imaging probe and then detected through streptavidin. Maldi-MS was used to confirm that the chemical entity synthesized was correct as seen in Figure 4.2. As shown in Figure 4.3, there are strong flouresence signals on the Bio5 targeted biofilm composed of *E.coli, S. mutans* and *S. aureus* after 60 mins incubation, while on the control group made of biotin-strepavidin , only few background signals. The biotinylated probe Bio5 was also assesed with human oral cells. Figure 4.4 shows that there is no binding for biotinylated Bio 5

biofilm probe (left) to human oral cells, while on the control group, made of biotinylated oral cancer probe, there are strong binding signals shown up (right).

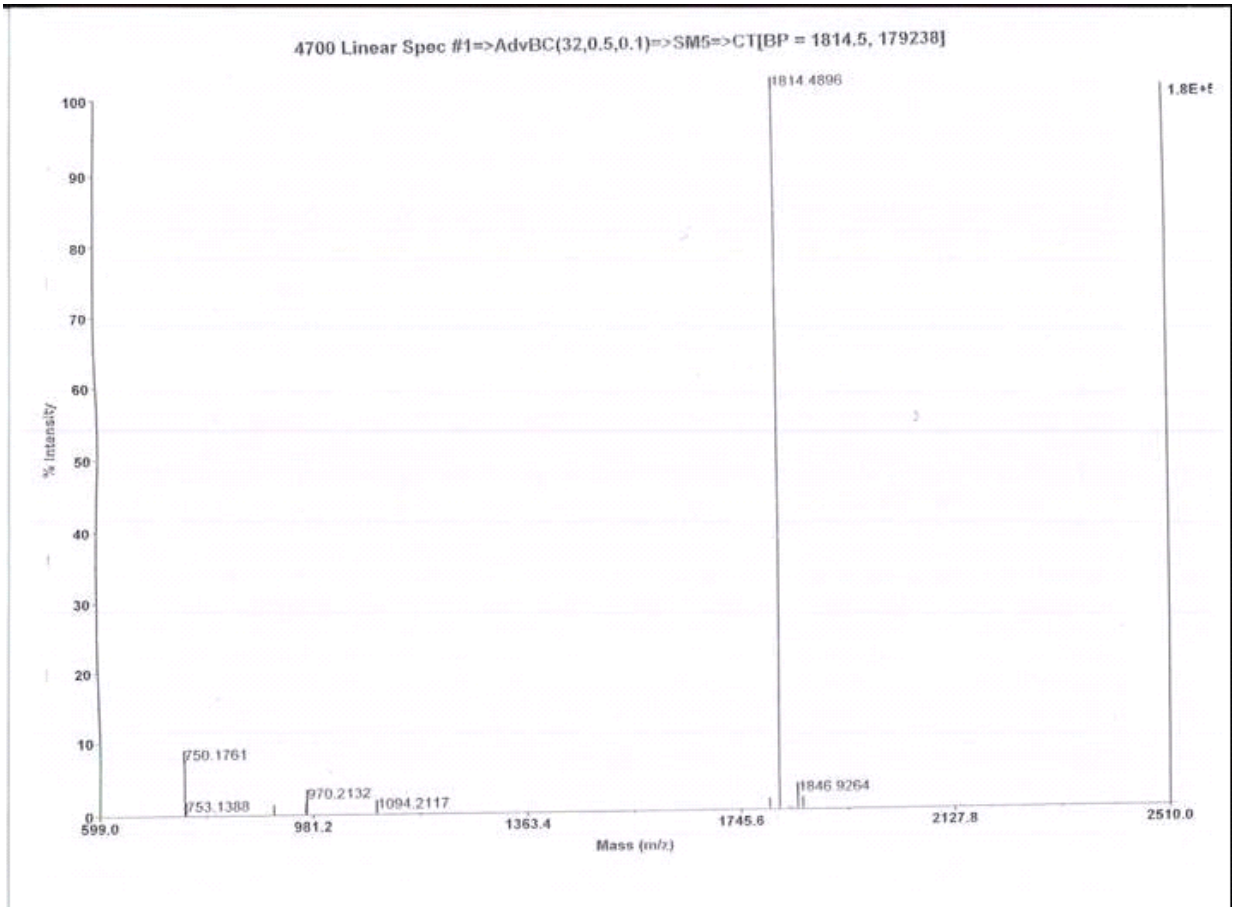


Figure 4.2: MS analysis of Bio5 with MW of 1814.4806

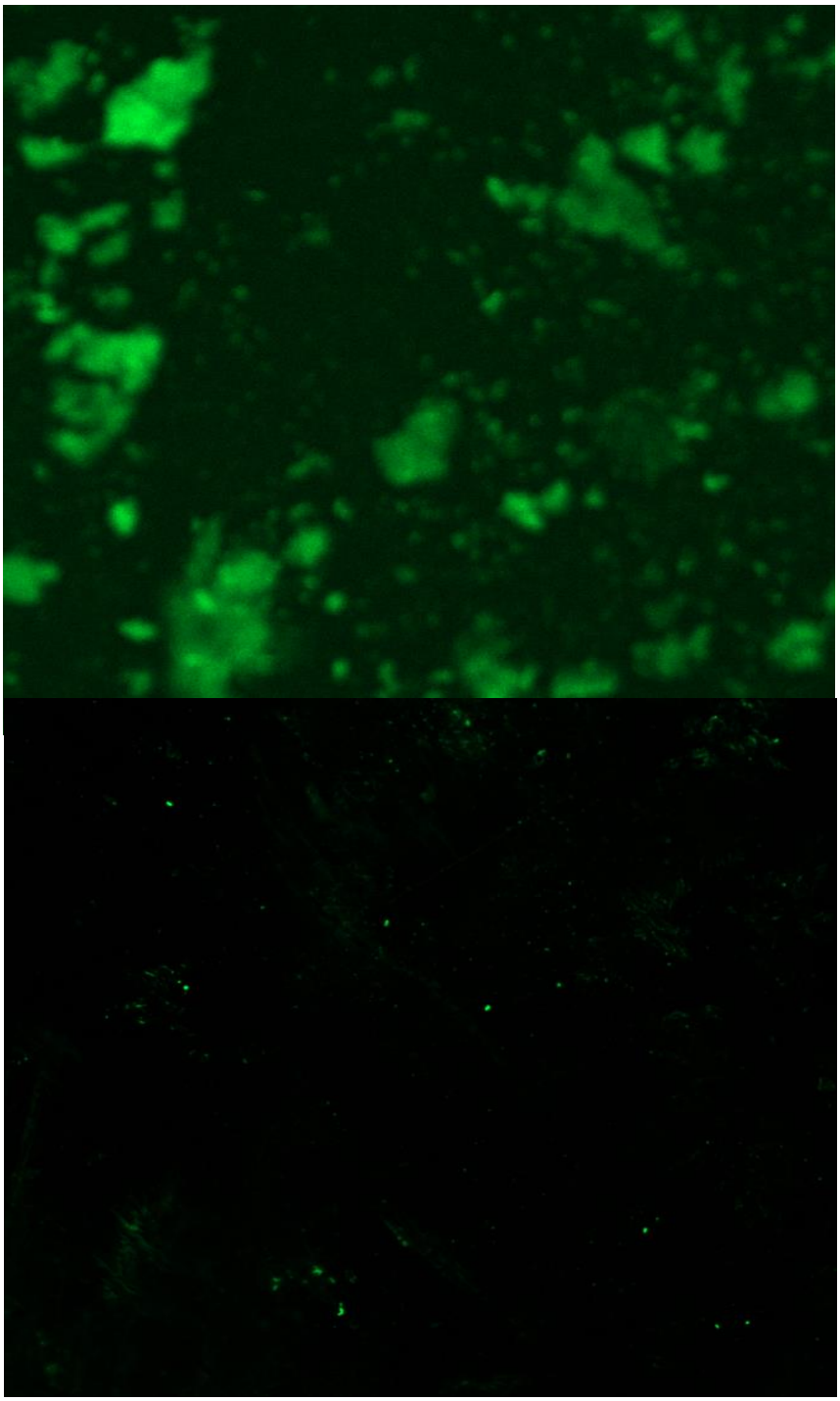


Figure 4.3: CLMS images of 14-day biofilm of *E.coli, S. mutans* and *S. aureus* exposed to biofilm probe Bio5. After 60 minutes incubation, there are strong fluorescence signals on the Bio5 targeted biofilm (top); while on the control made of biotin-streptavidin (bottom), only some background signals were seen.

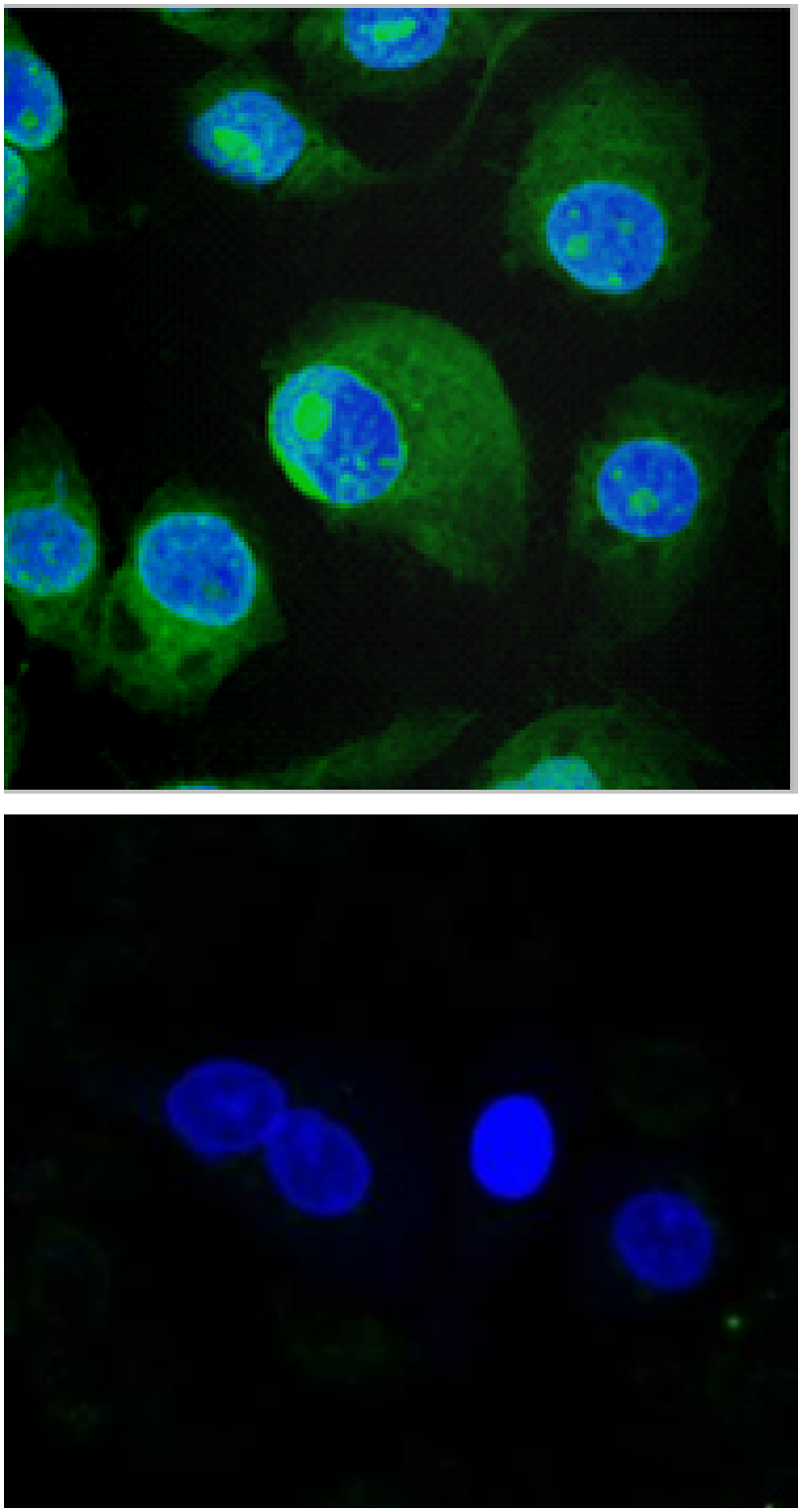


Figure 4.4: CLSM images of biofilm probe Bio5 exposed to oral cancer cells HSC3. There is no binding for biotinylated Bio 5 biofilm probe (bottom); while on the control group, made of biotinylated oral cancer probe, there are strong binding signals shown up (top)

4.5. Discussion:

Targeting probe to biofilm bacteria may provide new techniques of microbiology to initially detect biofilm infection in human tissues. Routine microbiological examinations are important and reliable for diagnosis of infections, but somehow less sensitive for biofilm detection. Therefore new techniques of microbiology on biofilm should be introduced as efficient complements of routine microbiology or part of the novel routine methods in hospitals for diagnosis of biofilm infection. Optical imaging is useful as a non-invasive preclinical imaging tool to evaluate or screen biofilm. Optical probe is simpler to develop technically, non-radioactive and relatively inexpensive. Through high-throughput screening assay, we identified four ligands, which are able to binding to 14-day multispecies biofilm at 10 um after 30 minutes incubation. To further investigation the probe's specificity, we incubate biofilm probe with human oral cancer cells HSC3 for one hour at % CO_2, 50 RPM. Biofilm probe do not bind to human oral cancer cells, while on the control group, made of biotinylated cancer binding ligand, shows stronger fluorescent binding signals (Figure 4.4). Work is under way to evaluate the binding ability on the real biofilm samples, such as biliary stent, urinary catheters, false teeth and etc. Although the biofilm probe reported here have the broad-spectrum of binding ability to bacteria, and cannot differentiate the bacterial species, they still can be use as the initial screening tool to detect the bacterial biofilm infection if proved effective on human real biofilm specimens.

One of the big concern of using peptides as the targeting probes is their stability *in vivo*. The biofilm probe reported here are made of D-amino acid, small molecules and L-amino acid, therefore are expected to be more resistant to protein enzyme digestion. Zonal

inhibition assay indicated these biofilm probes are not antibiotics, so the chance of creating resistant strain is less. So far, we have not yet to identify the ligand-receptor interaction between the binding ligand and the biofilm bacteria. It is reasonable to assume that these biofilm probes don't' bind to bacterial pili, since they bind both to gram positive and gram negative bacteria. We plan to incubate these biotinylated biofilm probes to both gram positive and gram-negative bacteria and following with streptavidin Nano gold conjugation to find out the binding site under TEM and SEM.

Chapter 5. Generation of all in one targeting theranostics against Biofilm

5.1. Abstract:

Background: Using combinatorial library techniques, we identified four biofilm ligands, Bio 4. *In vitro* studies demented stronger biofilm targeting efficacy. Biofilm targeting specificity of Bio5was further confirmed on human oral cancer cells. Therefore, Bio 5 was selected to construct targeting photosensitizer delivery vehicles to test the theranostic concept through phototherapy. The **aim** of this study is to construct a biofilm-targeted supramolecular photosensitizer delivery vehicles for photodynamic ablation against biofilm. **Material & method**: Two telodendrimers PEG^{5k}-CA_4-Por_4 & Male-PEG^{5k}-CA_8 were synthesized separately. After mixed in water, the two telodendrimers co-self-assemble to form monodisperse nanomicelles. Biofilm ligand Bio4 was chemically conjugated to the surface of the micelles via maleimide ligation to form targeting NPs micelles. Targeting ability of Bio4 micelles was studied by incubating multiple species of biofilms of *E.coli, S. mutans* and *S. aureus* in PBS and visualized under confocal laser scanning microscope. The biofilm bacterial killing ability of Bio4 conjugated micelles was then studied by the treatment of the biofilm with 660 nm laser irradiation. Biofilm cultured in 96-well plates were dispersed after sonication for 10 min and number of living bacteria were determined by gradient dilution and plate counting method. Treated biofilms were also visualized under CLSM after live & dead staining. **Result**: the size of targeting Bio4 micelles were 20.56± 4nm in diameter. Multiples species of biofilm exhibit red fluorescence under 561 nm excitation after targeting nanoparticles were added. In contrast, biofilm incubated with no targeting micelles showed much weaker fluorescence. After 6 min irradiation, the Bio 5 micelles exhibit a drastic biofilm inactivation effect (2.93 log10 reduction) when compared to non-targeting micelles. **Conclusion**: NPs conjugated with Bio5 might be able to target biofilm cells and exhibits biofilm killing ability *in vitro*. More experiment will be performed to get conclusion.

5.2 Introduction

Photodynamic therapy (PDT), in which the photosensitizers are activated by light irradiation to generate reactive oxygen species (ROS), has drawn tremendous interests for its antimicrobial applications. This light-initiated ROS are highly reactive and can damage cellular components, for example, cell membranes, nucleic acids, protein and lipids, thus inducing cell inactivation. The multi-target process of PDT provides an effective approach to avoid the resistance in biofilm and shows great potential for biofilm treatments. However, considering limited diffusion distance of ROS (<50nm) (58), accumulation of photosensitizers in or around bacteria is essential to conduct efficient antimicrobial PDT. Therefore, cationic photosensitizers and photosensitizer-cationic molecule conjugates have been developed to meet this need. Cationic porphyrin and cationic polyethylenenime (PEI)-Chlorin conjugated have been proven to be effectively bound to bacterial membranes and enhanced PDT performances was achieved. Nevertheless, the cytotoxicity of these molecules to mammalian cells cannot be ignored. These nonselective cationic molecules will not only bind to cell membranes, disrupting membrane integrity and functions, but also bring photosensitizers to cells, causing serious phototoxicity. It is still challenging to design a smart photosensitizer delivery vehicle which can cover the requirement for targeting bacteria as well as reducing damage to normal cells.

Nanoparticles, in definition are unltra-dispersed solid supramolecular structures with a submicrometer size ranging from 10 to 100 um. Different reported recipes for nanoparticles include a dispersion of preformed polymers, the polymerization of monomers, ionic gelation and coacervation of hydrophilic polymers, etc. The precise

control of the size of these nanocarriers allows them to travel systemically yet limit their crossing of healthy region of the organism with tight spacing of 15-30 nm. Recently, a new nanoplatform developed by my lab. It was reported as 'robust, smart and highly versatile all in one porphyrin based organic Nano construct that can be self-assembled from a novel hybrid amphiphilic polymer called teldodendrimer, comprised of dendritic cholic acid and porphyrin linked to a linear PEG. These porphyrin-based nanoparticle allows (i) efficient encapsulation of hydrophobic chemotherapeutic drugs, (ii) near-infra red fluorescent (NIRF) detection of the tumor based on the intrinsic fluorescence of porphyrins, (iii) efficient free radical and heat generation at tumor site upon activation with light for photodynamic therapy (PDT) and photothermal therapy (PTT), respectively, and (iv) convenient ligation of cancer-targeting ligands to the surface of the micelle for cancer-specific targeted delivery (29-33). This new porphyrin based nanoparticle when loaded with paclitaxel (PTX) and decorated with ovarian cancer binding ligands (discovered through combinatorial library technique), exhibit superior anti-tumor efficacy and lower systemic toxicity profile in nude mice bearing ovarian cancer tumor xenograft when compared with equivalent doses of non-targeted PTX nanoparticles as well as clinical PTX formulation (32,33). This versatile porphyrin/cholic acid hybrid telodendrimer can self-assemble in aqueous solution by design, thereby integrates a variety of imaging and therapeutic function that includes imaging (near-infrared fluorescent imaging (NIFI), positron emission tomography, magnetic resonance tomography (MRI), dual modal PET-MRI, PTT and PDT as well as targeted drug delivery. In this study, we explore the potential targeting efficacy after novel biofilm

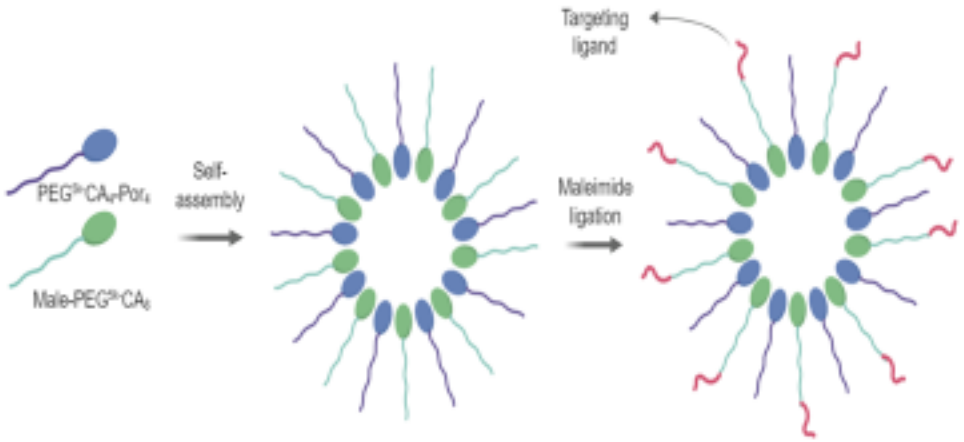
targeting ligand, Bio4, is conjugated to this novel nanoplatofrm called nanoporphyrin (NP) as “nanoagents” for diagnosis and treatment of biofilm.

5.3. Methodology

5.3.1. Materials: bifunctional polyethylene glycol was purchased from Rapp Polymere (*Tübingen*, Germany). Cy5.5 Mono NHS ester was purchased from Amersham Biosciences (Piscataway, NJ). Cholic acid, MTT [3-(4, 5-dimethyldiazol-2-yl)-2, 5 diphenyl tetrazolium bromide] and all other chemicals were purchased from Sigma-Aldrich.

5.3.2. Generation of targeting NPs: The traditional porphyrin/CA hybrid telodendrimer (PEG^{5k}-Por_4-CA_4), and the ligand conjugated porphyrin/CA hybrid telodendrimer (L-PEG^{5k}-Por_4-CA_4), were synthesized through solution-phase condensation reaction from MeO-PEG-NH_2 and N_3-PEG-NH_2 using peptide chemistry (Figure 5.1). In brief, Fmoc)Lys(Fmoc)-OH(3 eq.) was coupled on the N terminus of PEG using DIC and HOBt as coupling reagents until a negative Kaiser test result was obtained, thereby indicating completion of the coupling reaction. PEGylated molecules were precipitated by adding cold ether and then washed with cold ether twice. Fmoc groups were removed by the treatment with 20% (v/v) 4-methylpiperidine in dimethylformamide (DMF), and the PEGylated molecules were precipitated and washed three times by cold ether. White powder precipitate was dried under vacuum and one coupling of (Fmoc)Lys(Fmoc)-OH and one coupling of (Fmoc)lys(Boc)-OH were carried out, respectively, to generate a third generation of dendritic polylysine terminated with four Boc and Fmoc groups on one end of PEG. CA NHS ester were coupled to the terminal end of dendritic polylysine after the removal of Fmoc with 20% (v/v) 4-methylpiperidine and the removal of Boc

groups with 50% (v/v) trifluoroacetic acid in dichloromethane, respectively. The telodendrimer solution was filtered and then dialysed against 4 l water in a dialysis tube with MWCO of 3.5 KDa; reservoir water was refreshed completely four times in 24 h. Finally, the telodendrimer was lyophilized. Alkyne modified Bio 4 ligand (imipqwa-Ebes-K-alkyne) was synthesized via solid-phase synthesis on Fmoc-Rink Amide MBHA Resins using the standard Fmoc chemistry as described previously (32,33). 5-hexanoic acid was coupled onto the ε-amino group of lysine on the peptide. Alkyne modified ligands was conjugated to the N_3-PEG^{5k}-CA_8 telodendrimer *via* Cu^I catalyzed cyloaddition. The conjugation was confirmed by the amino acid analysis (AAA). Preparation of NP In all, 20 mg porphyrin-telodendrimer or ligand-porphyrin-telodendrimer were dissolved in 1 ml PBS followed by sonication for 10 min to form NPs. In order to make CNP, 20 mg total amount of PEG^{5k}-Cys_4-Por_4-CA_4 was dissolved in 1 ml PBS to form micelles and then sonicated for 10 min.



Schematic illustration of Bio 4 decorrected PEG^{5k}-CA$_4$-Por$_4$/Male-PEG^{5k}-CA$_8$ nanomicelles. Two telodendrimers PEG^{5k}-CA$_4$-Por$_4$ & Male-PEG^{5k}-CA$_8$ were synthesized separately. Once mixed in water (or PBS), the two telodendrimers can co-self-assemble to form monodisperse nanomicelles. Peptide ligand will chemically attach to the surface of the micelles via maleimide ligation. After incubated with the biofilm, the targeting ligand decorated micelles will accumulate in the bacteria. And followed by XXXmin the light shining (wavelength: XXXXnm), which leading to the death of bacteria.

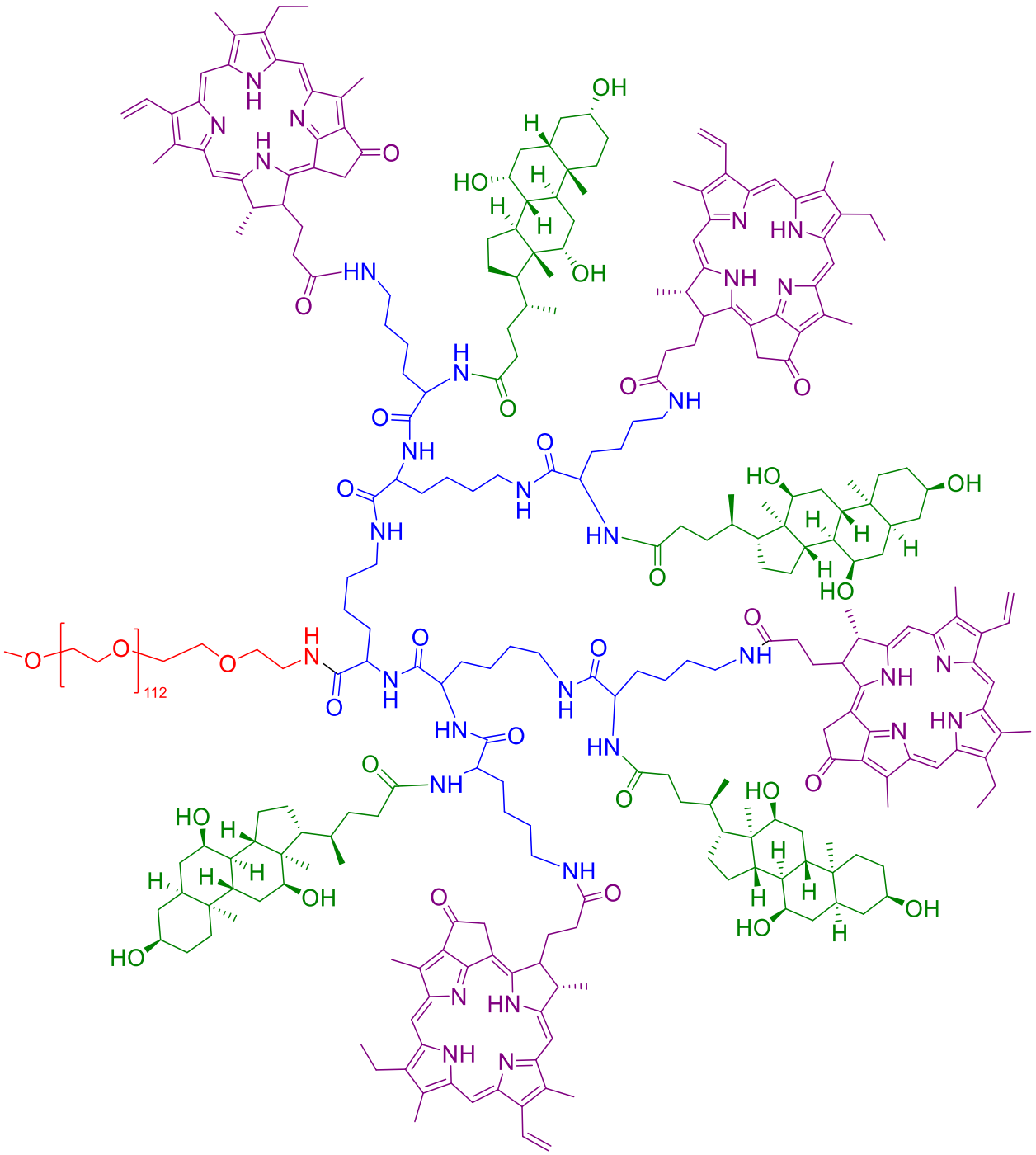


Figure 5.1: Schematic illustration of generation of PEG5k-CA8 – Porphyrin telodendrimer

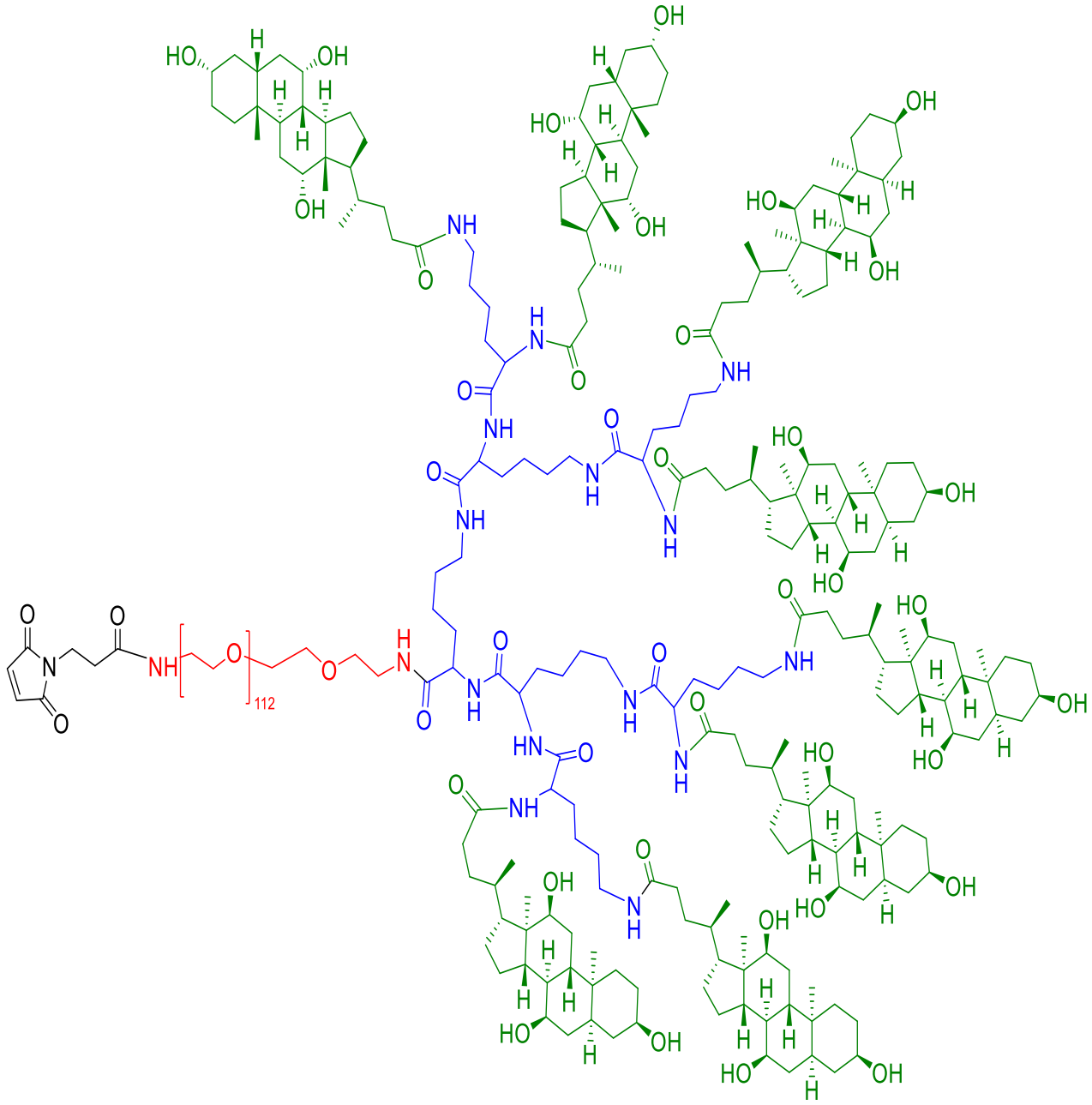


Figure 5.2: Schematic illustration of generation of N3-PEG5k-CA8 telodendrimer

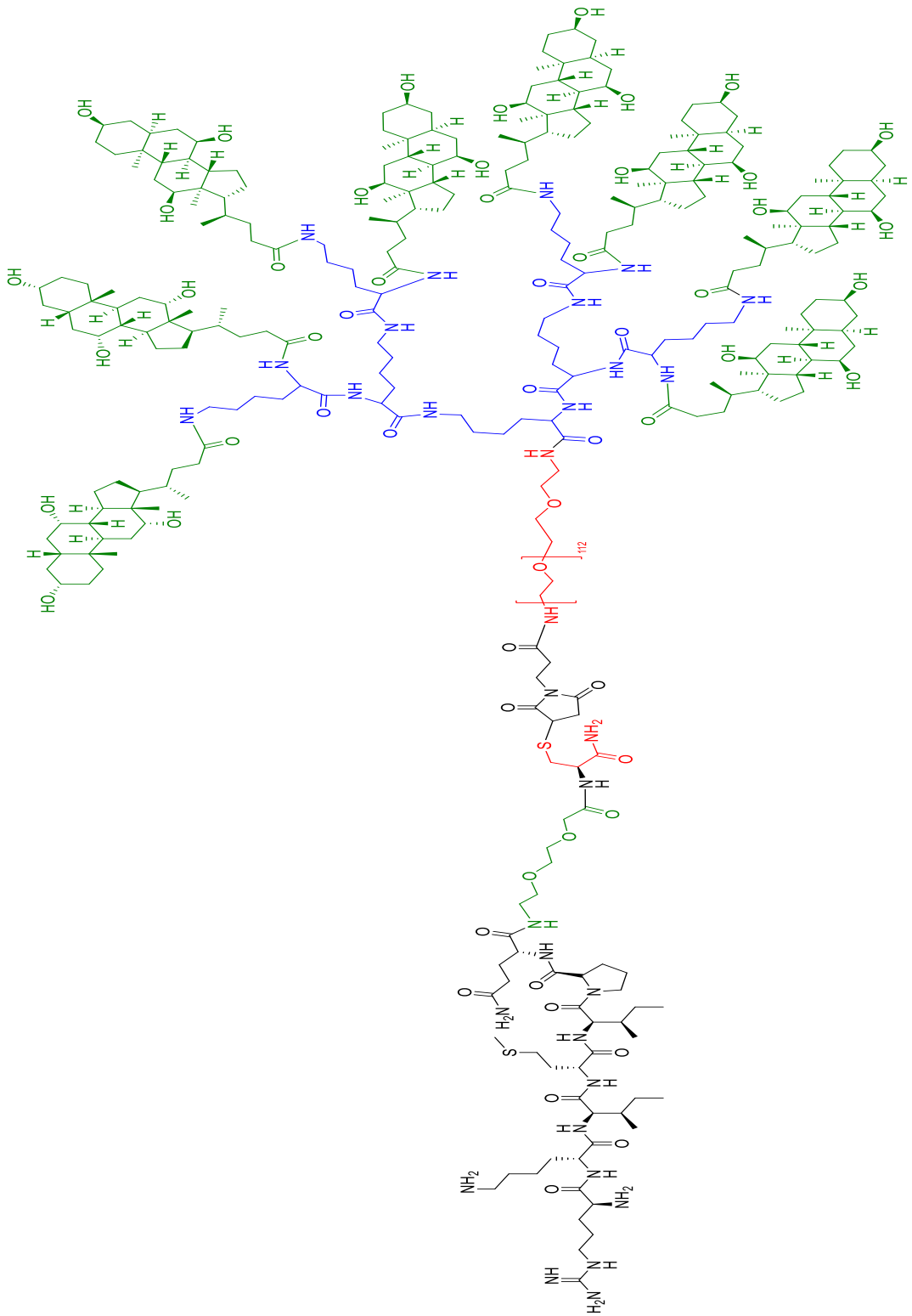


Figure 5.3: Schematic illustration of biofilm ligand Bio5 conjugated supramolecular micelles

5.3.3. Characterization of targeting NPs: In all, 20 mg porphyrin–telodendrimer were first dissolved in CHCl3/MeOH, mixed and evaporated on rot vapor to obtain a homogeneous dry polymer film. The film was reconstituted in 1 ml PBS, followed by sonication for 30 min, allowing the sample film to disperse into NP solution. Finally, the NP solution was filtered with a 0.22-μm filter to sterilize the sample. Dynamic light scattering (DLS) was used to measure the size of nanoparticles synthesized. The absorbance spectra of ligand porphyrin/CA hybrid telodendrimer was measured through UV-spectroscopy.

5.3.4. Photosensitizers and light sources: The photosensitizers used in this studies are the Bio4 conjugated nanoporphyrins generated. A light-emitting diode (LED) lamp, emitting in the red spectrum with a peak at 628nM.

5.3.5. Targeting photodynamic therapy on bacterial biofilm: Bacterial biofilm will be generated by inoculating *E.coli, S.mutans* and *S. aureus* in to 96 well tissue plates and incubation for 14 days under 37º C, 50 RPM. The fresh media will be replaced every three days. After gently washed with PBS to remove non-adherent bacteria, the experimental conditions are (i) biofilms sensitized with targeting nano porphyrin in darkness for one hour, two hours and four hours ; (ii) biofilms sensitized with targeting nano porphyrin in darkness at different concentration (iii) biofilms sensitized with targeting nano porphyrin were treated with diode laser at different output power; (output power from 100 mW to 500 mW), (iv) biofilms sensitized with targeting nano porphyrin in darkness and then exposed to diode laser at different time intervals (from 1 to 10 mins). The controls consisted of (i) biofilms treated with non-targeting nano porphyrin, (ii) biofilms treated only with the diode laser.

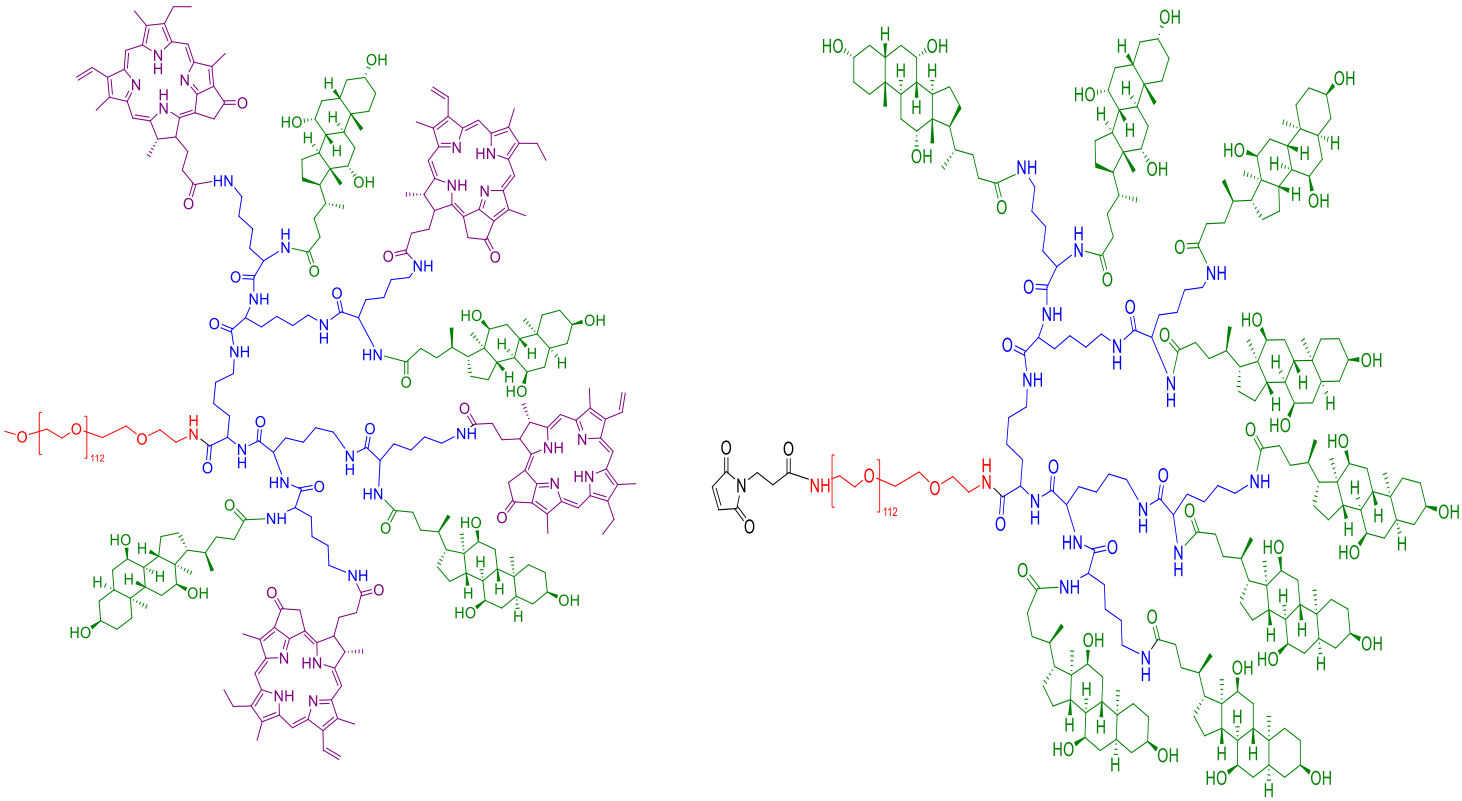
5.3.6. CLSM imaging to visualize live & dead bacteria: after treatment, biofilms on the 96 well tissue plates are washed three times with distilled water to remove loose bacteria and then stained using the LIVE/DEAD *BacLight* bacterial viability kit (Molecular Probes, Eugene, OR). In this system, live bacteria stain with Syto 9 to produce a green fluorescence whereas bacteria with compromised membranes stain with propidium iodide to produce a red fluorescence. Images of the double-stained biofilms were acquired using a Leica TCS-SL filter-free spectral confocal laser scanning microscope. The CLSM images were analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA) to enumerate viable and dead bacteria.

5.3.7 Viable accounts for bacterial enumeration*:* briefly, after treatment with phototherapy, biofilm bacteria in 96-well plates were sonicated for 10 mins. After a serial dilution of biofilm bacteria with PBS, 100 ul of aliquot are plated in TSA agar. The colonies of bacteria grown on the agar are counted. The number of live bacteria (viable accounts) are transformed into logarithms of 10 and expressed as the mean (log 10 CFU/cm2 $\pm$ standard deviation. Exploratory data analyses including summary statistics are performed using Minitabb software.

5.3.8. Statistical analysis: Statistical analysis was performed by Student's *t*-test for two groups, and one-way analysis of variance for multiple groups. All results were expressed as the mean±s.d. unless otherwise noted. A value of $P<0.05$ was considered statistically significant.

5.4. Results

Using the method published in my previous lab, we successfully synthesize the Two telodendrimers PEG^{5k}-CA_4-Por_4 & Male-PEG^{5k}-CA_8 separately. After mixed in water, the two telodendrimers co-self-assemble to form monodisperse nanomicelles. Biofilm ligands Bio4 was chemically conjugated to the surface of the micelles via maleimide ligation as shown in figure 5.6. The size of targeting nanoparticles was 20.56 +6.14 compare to non-targeting one with 21.89+6.91 (Figure 23).



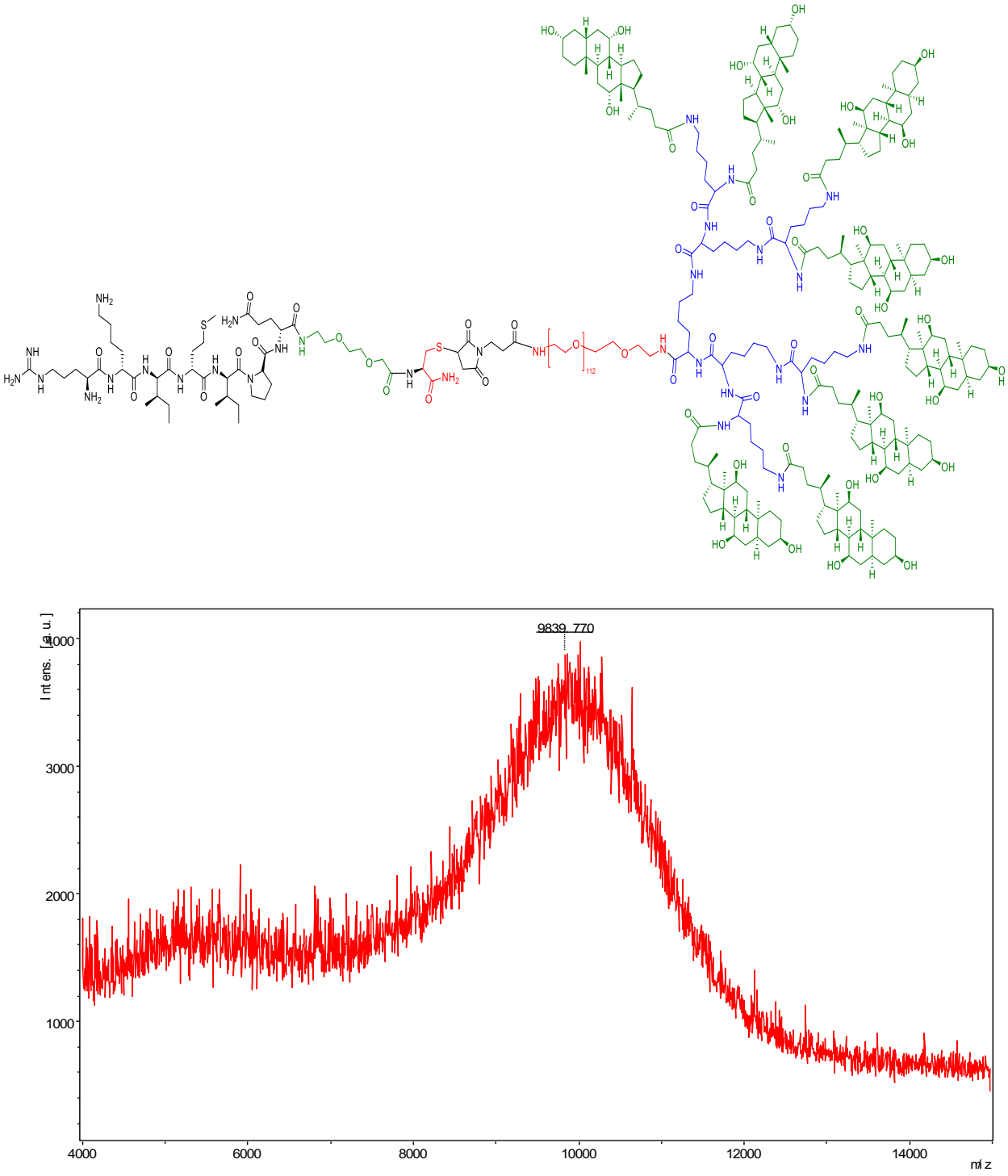
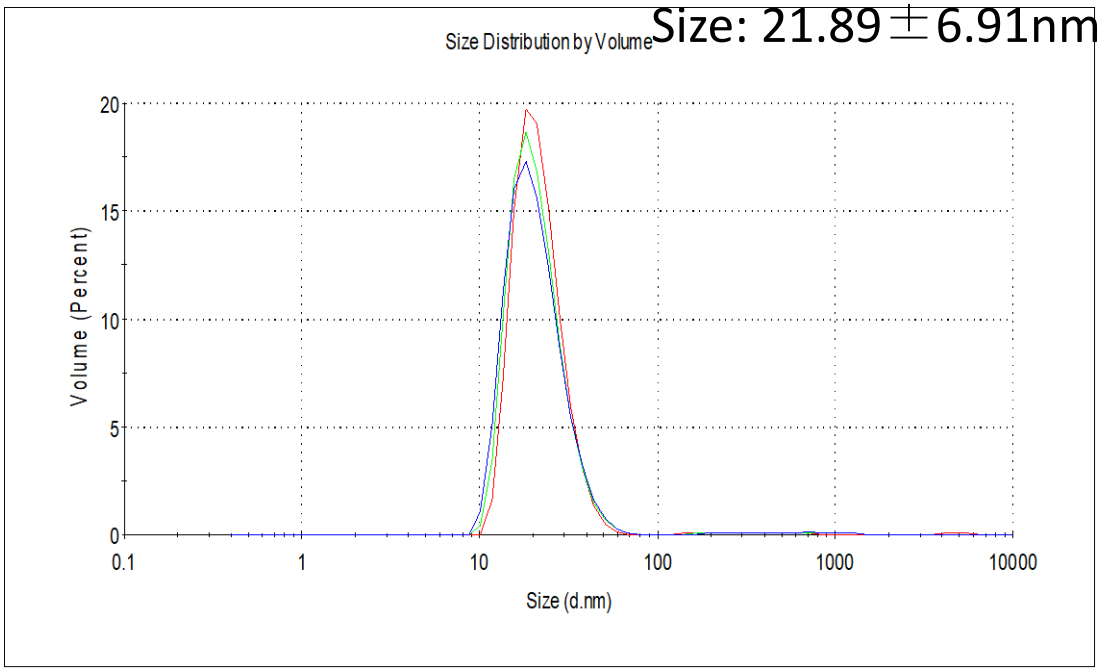


Figure 5.4: Structures and MS spectra of PEG5k-CA4-Por4 & Mal- PEG5k-CA5



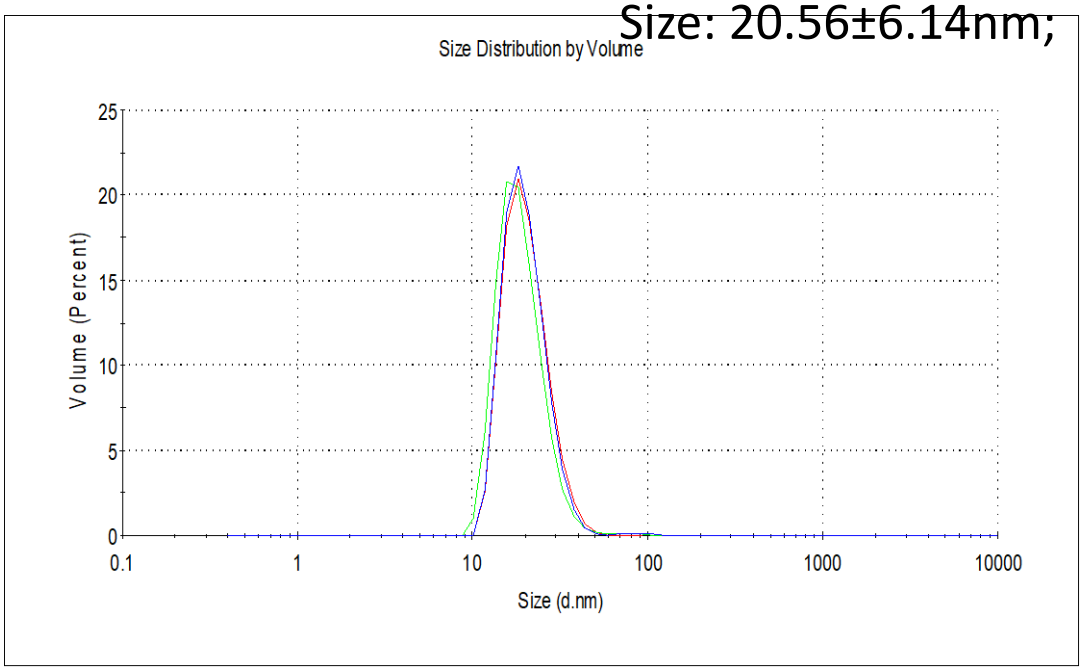


Figure 5.5: Size distribution of targeted NPs. The average size of targeted NPs was 20.56 +6.14, while the average size of non-targeted NPs was 21.89+6.91.

The targeting nanoparticles Bio4 were then added to 14-day multispecies of Mixed biofilm created on 96 well tissue plates, composed of *E.coli, S.mutans* and *S.aureus* for four hours and incubated for 2 hours. After 5x wash with water, the biofilm were visualized under CLSM. As shown in Figure 5.6, there are red auto fluorescent signals from the intrinsic porphyrin in the targeting nanoparticle group while no signal was visualized on negative control, composed of nanoparticles only. This shows that the targeting nanoparticles have superior biofilm targeting and penetration ability than the control group

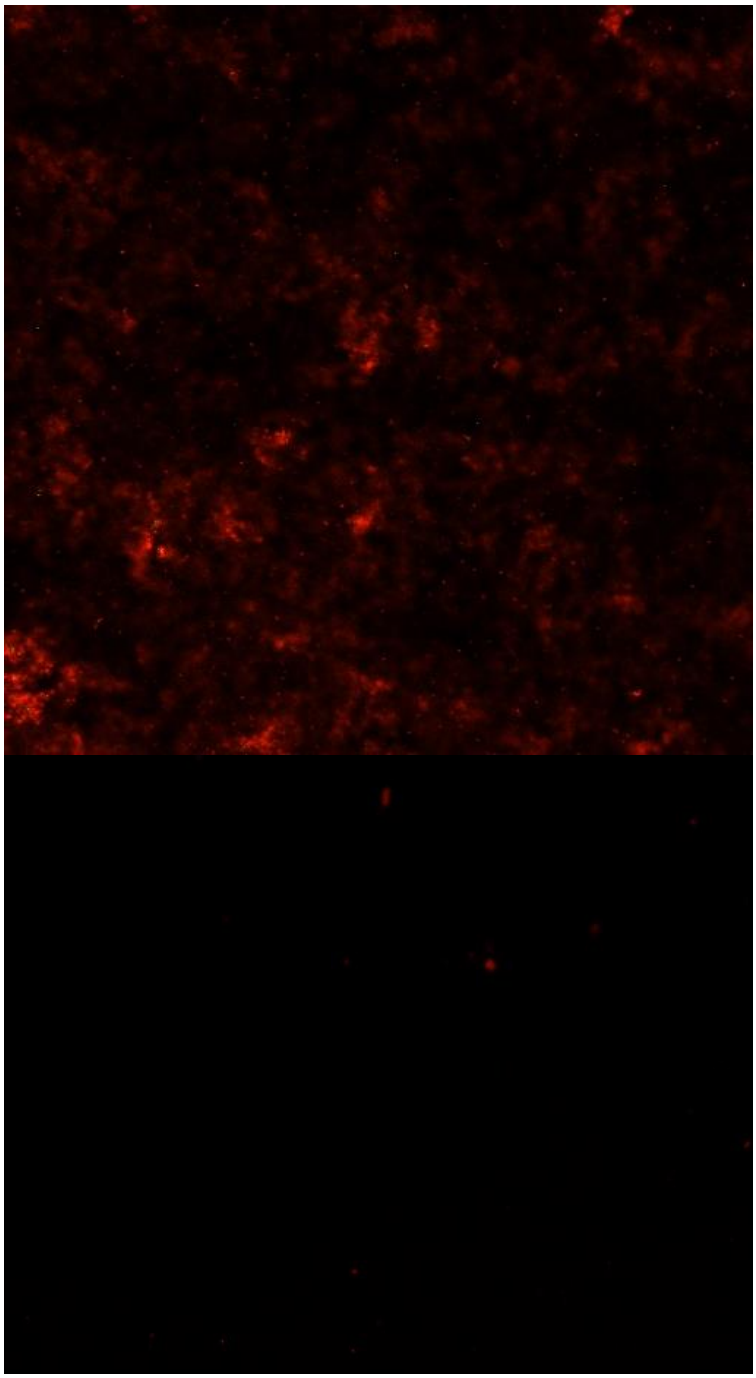


Figure 5.6: Confocal images of targeted NPs exposed to 14-day multispecies biofilm. There are red nanoparticles signals shown on the targeting group (top); while only background signals shown on the control group (bottom).

The 14-day multispecies biofilm were incubated with 125 ug/ml of targeted NPs and incubated for two hours. After washed with PBS,

biofilm bacteria were challenged with phototherapy for 5 minutes. As shown in Figure 5.7, there is significant photothermal change in the targeting nanoparticle phototherapy group where the temperature increased by 23.1C degree.

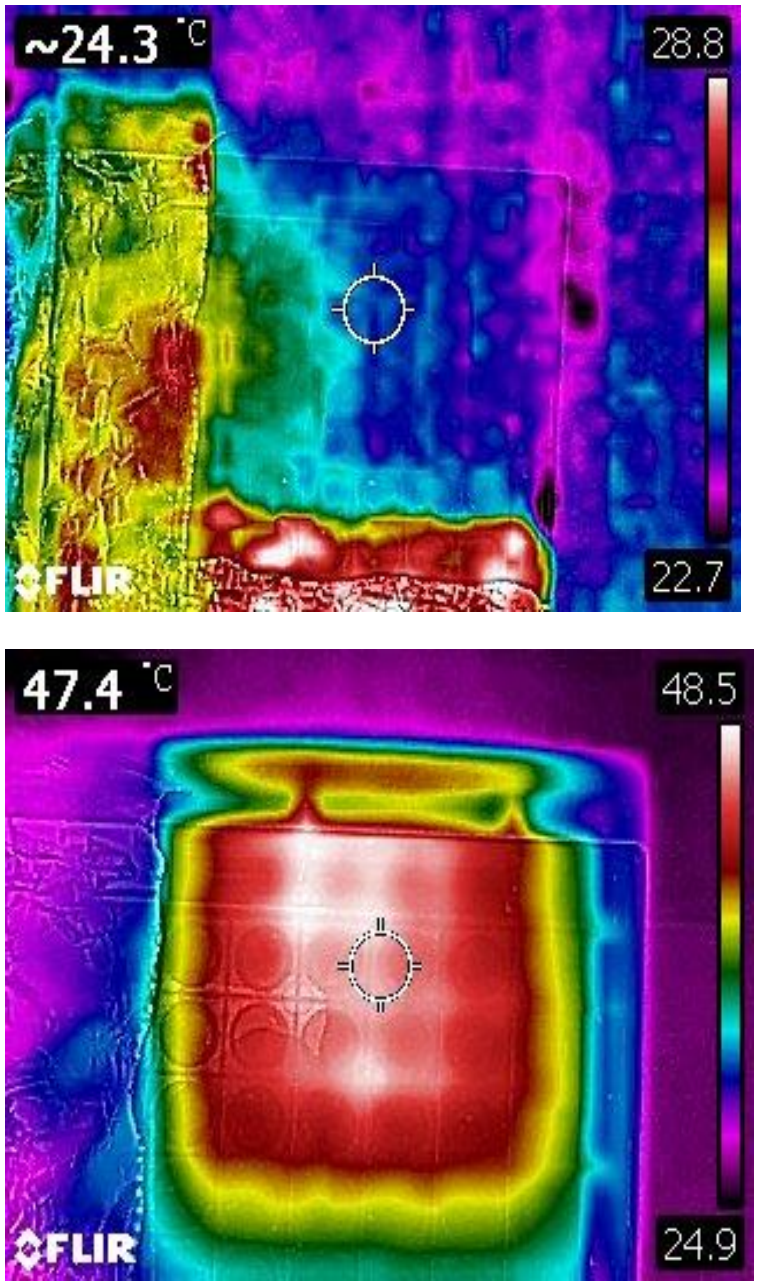


Figure 5.7: Temperature changes of biofilm plates after irradiation. The temperature was increased 47.4 C after irradiation (right) compared to prior irradiation (left).

There are 2.93 log 10 reduction of bacterial number after irrigation compared with control group; and there are 2.3 log 10 reduction of bacterial number compared with irradiation only. The results are summarized in Figure 5.8.

		Log number	Mean+ SD
• Control:	3.4x10^7	7.5	
	2.8x10^6	6.4	6.83+0.59
	3.9x10^6	6.59	
• Light irrigation only:	5.1x10^6	6.7	
	4.4x10^5	5.64	6.20+0.53
	1.9x10^6	6.27	
• Targeting irrigation:			
	3x10^3	3.4	
	5.6x10^3	3.74	3.9+0.59
	3.7x10^4	4.56	

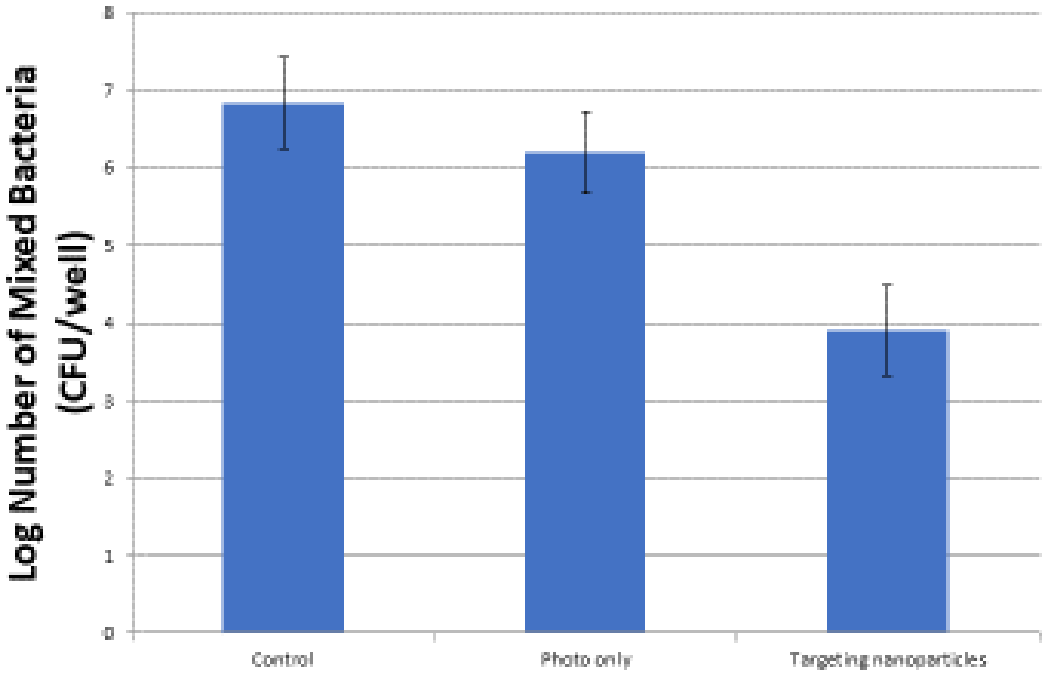


Figure 1: Summary of numbers of biofilm bacteria after irradiation. There are 2.93 log 10 reduction of bacterial number after irrigation compared with control group; and there are 2.3 log 10 reduction of bacterial number compared with irradiation only. $p < 0.05$.

The biofilm bacteria killing ability was further confirmed by live & dead stain. As shown in Figure 5.9, there are drastic differences in the therapeutic effect of the targeting Np vs

the non-targeting NP. Dead bacteria count on the targeted NPs after irradiation is significantly higher than the control group where non targeting NP was used.

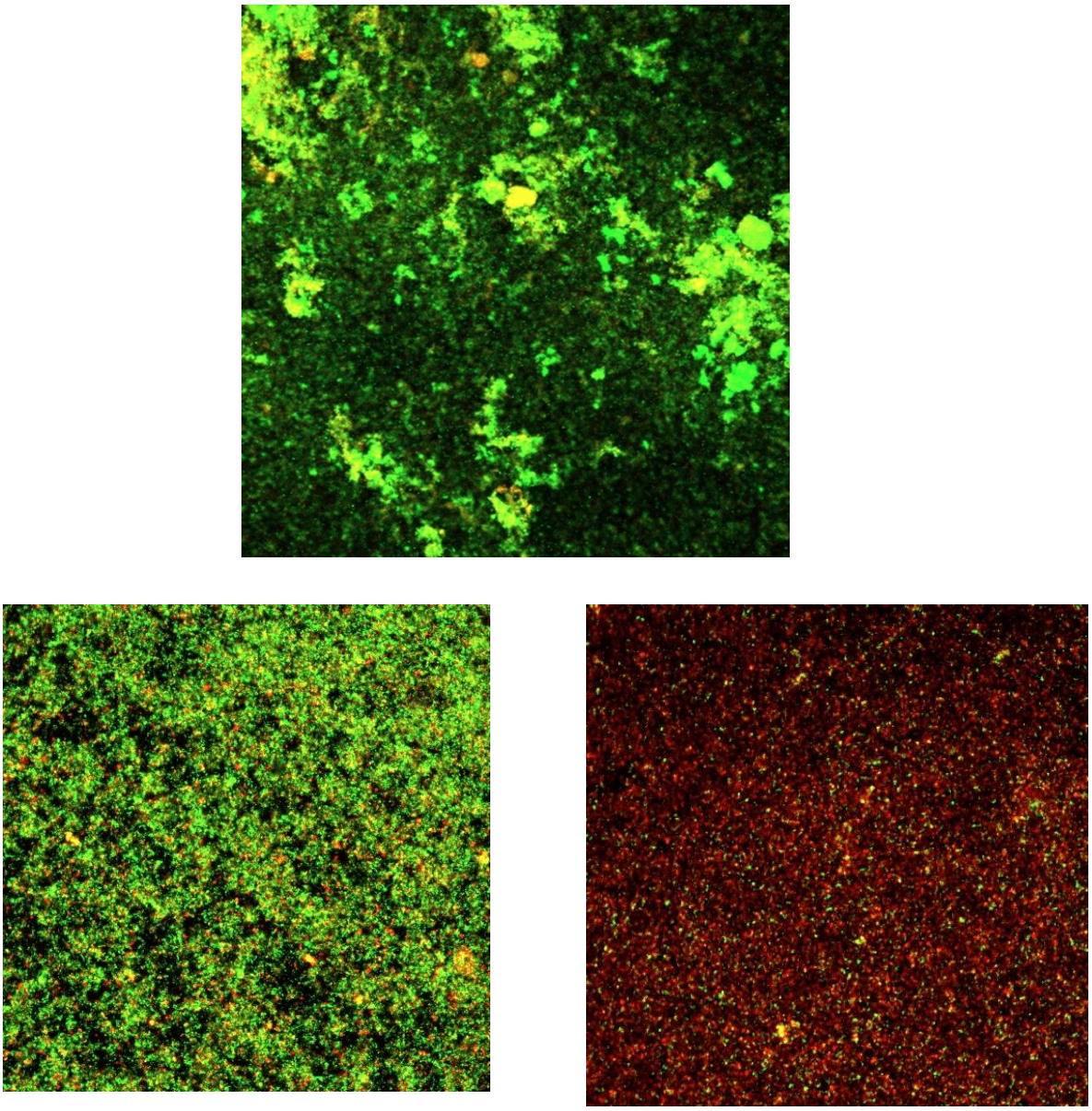


Figure 5.9: Confocal images of 14-day multispecies biofilm after irradiation. There are many dead bacteria (red) in the targeted group after irradiation (right); a few dead bacteria after irradiation only (middle); and control group without irradiation with all ligands.

5.5. Conclusion: NPs decorated with Bio5 might be able to target biofilm cells and exhibit biofilm killing ability *in vitro*. This experiment has been only performed for one time; more experiments will be performed to get conclusion.

5.5. Discussion

PTT and PDT are promising strategies for the treatment of bacterial infectious diseases, especially for the inhibition of drug-resistant development and biofilms formation. NPs used for PTT and PDT possess many advantages such as high PS loading capacity and controlled release to increase the antibacterial properties. However, undesired damage to normal cells may be caused due to the nonselective nature of PDT. Therefore, targeted PDT is preferred on one hand to enhance antimicrobial effects and on the other hand to reduce cytotoxicity to normal cells. For this purpose, we performed high throughput screening assay to search for specific biofilm binding ligands. Finally four biofilm ligands were identified for further studies. Bio5 was selected to construct bacteria-targeted supramolecular photosensitizer delivery vehicles due to it possess stronger biofilm bacterial binding ability (10 μM), in addition, Bio5 didn't bind to human epithelial cells, endothelial cells, fibroblasts and granulocytes, as well as human oral cancer cells. While NPs-Bio4 produced significant outcome for PDT and PTT-mediated killing of biofilm bacteria after duration of incubation with the biofilm for 2 h, this means that the bacteria binding ligand, if conjugated with nanocarriers, do exert biofilm bacterial killing effect. Therefore, investigation of targeting Bio5-NPs's behavior on individual bacterial biofilm has emerged to be a necessity, including *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *E. coli*, *E. Faecalis*, *K. Pneumonia*, *S.mutans*, *C. amalonaticus*, *P. gingivalis*. In the meantime, the parameters of biofilm bacteria killing efficacy, such as optimal incubation time, power and time of irradiation, concentration of targeting NPs, need to be further clarified.

The advantages of this study is the discovery of broad-spectrum biofilm bacteria ligands, including gram positive, gram negative, aerobic and anaerobic bacteria. The bacteria used

for the screening cover almost most antibiotic resistant bacteria, such as *S. aureus*, *P. aeruginosa*, *E. coli*, *E. Faecalis*, *K. Pneumonia*, *C. amalonaticus*,, although these strains are not super bugs. In the future, if these ligands are confirmed possess the capability to bind the antibiotic resistant strains, they will be much more valuable to become a powerful tool to combat the super bugs. It is interesting to note that biofilm bacteria possess stronger binding ability on the compound beads compared to planktonic bacteria of same strains and same density (data not shown).

Two factors should be explored to enhance the biofilm bacterial killing efficiency which are the penetration depth and stability of the NPs. The four ligands discovered are composed of mix D and L amino acid and small molecules, thus expecting to be resistant to proteinase digestion. The size of targeting NPs, Bio 4-NPs developed here are 20.56 $\pm$6.14. According to the literatures, the optimal size for NPS penetration to biofilm is <50 nm. We are planning to perform experiment to observe the depths of targeting NPs in biofilm in the different time intervals.

In summary, the data presented here demonstrate the treatment with small molecules, such as Bio5, hold promise for development as anti-biofilm therapeutics. As we struggle to maintain effective antimicrobial therapies with the global threat of emerging resistance, novel strategies to the pathogens will need thoroughly explored.

Chapter 6: Conclusion and future work:

In this project, we have used combinatorial library technology to identify biofilm specific ligands for the improvement of screening and diagnostic techniques in parallel with the development of new therapeutic molecules against biofilm bacteria. Four broad-spectrum bacterial ligands were identified for possessing the property of specifically binding to bacteria, *including S.aureus, E.faecalis*, *S.mutans ,P.aeruginosa* , *K. pneumonia , C. amlonaisus*, *P.gingivitis* but not binding to normal human oral cells. MTT assays indicated that these four ligands have no cytotoxicity to human epithelial cells at up to 50 uM. Zonal inhibition assay indicated these four ligands do not have bactericidal effects, therefore, they are not antibiotics. These four bacterial ligands were selected for the development of biofilm probes.

Although four peptides bind to bacteria strongly on the solid support of polystyrene beads, we need to evaluate their bacterial targeting ability in solution form. Therefore, peptides were synthesized in solution form to generate biotinylated optical imaging probe and then detected through streptavidin. There are strong flouresence signals on the Bio5 targeted biofilm composed of *E.coli, S. mutans* and *S. aureus* after 60 mins incubation; while on the control group made of biotin-strepavidin only, only background signals were observed. Therefore, Bio5 was selected to construct bacteria-targeted supramolecular photosensitizer delivery vehicles for photodynamic ablation against biofilm.

PTT and PDT are promising strategies for the treatment of bacterial infectious diseases, especially for the inhibition of drug-resistant development and biofilms formation. NPs used for PTT and PDT possess many advantages such as high PS loading capacity and controlled release to increase the antibacterial properties. However, undesired damage to

normal cells may be caused due to the nonselective nature of PDT. Therefore, targeted PDT is preferred on one hand to enhance antimicrobial effects and on the other hand to reduce cytotoxicity to normal cells. For this purpose, we performed high throughput screening assay to search for specific biofilm binding ligands. Finally four biofilm ligands were identified for further studies. Bio5 was selected to construct bacteria-targeted supramolecular photosensitizer delivery vehicles due to it possess stronger biofilm bacterial binding ability (10 μM), in addition, Bio5 didn't bind to human epithelial cells, endothelial cells, fibroblasts and granulocytes, as well as human oral cancer cells. Initial study demonstrated that NPs decorated with Bio5 might be able to target biofilm cells and exhibit biofilm killing ability *in vitro*. This experiment has been only performed for one time; more experiments will be performed to get conclusion. Therefore, investigation of targeting Bio5-NPs's behavior on individual bacterial biofilm has emerged to be a necessity, including *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *E. coli*, *E. Faecalis*, *K. Pneumonia*, *S.mutans*, *C. amalonaticus*, *P. gingivalis*. In the meantime, the parameters of biofilm bacteria killing efficacy, such as optimal incubation time, power and time of irradiation, concentration of targeting NPs, need to be further clarified.

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