SurfSAFE

SCIENTIFIC WORKSHOP 1:

Characterization of non-biotoxic, antimicrobial, anti-adhesive and biomimetic surfaces

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Abstract Booklet

PRESENTED WORKS

The impact of different biofilm architectures on Legionella proliferation

Ana Rosa Silva, Ana Pereira and Luis F. Melo

LEPABE - Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal
arms@fe.up.pt (A.R.S.); aalex@fe.up.pt (A.P.) and lmelo@fe.up.pt (L.F.M.)

Legionella pneumophila is a waterborne pathogenic bacterium, ubiquitous in natural and man-made water systems, such as cooling towers, humidifiers, premise plumbing and drinking water systems. It is responsible for outbreaks of Legionnaires' disease worldwide, being transmitted to humans through the inhalation of contaminated aerosols. Biofilms attached to the water systems surfaces assume a relevant role in *Legionella* colonization and proliferation. Several factors are known to affect biofilm build-up and, also biofilm colonization and proliferation by *Legionella.* Thus, it becomes very important to study the ecology of *Legionella* in association with biofilms.

The aim of this project is to study: (i) how *L. pneumophila* colonizes and proliferates in biofilms with different architectures, formed under different hydrodynamic, suspended particles and temperature conditions; (ii) how the resultant *Legionella* colonization patterns and biofilm structural properties affect the release and spread of *Legionella* after detachment of biofilm layers from the surfaces. For that, a *Pseudomonas fluorescens* biofilm will be formed under different conditions on Stainless Steel (SS) 316L coupons inside a Center for Disease Control (CDC) Biofilm Reactor. *Legionella pneumophila* will be added to the bioreactor under two different situations (3 and 12 days after biofilm formation). Samples will be taken for bulk water and biofilm analysis. Biofilm formation will be followed by confocal microscopy and special emphasis will be given to the visualization of *Legionella* position inside the biofilm, which will be accomplished by marking the bacterium with Fluorescent in Situ Hybridization (FISH) techniques. This work might provide a critical output towards prevention and implementation of control strategies regarding *Legionella* mitigation.

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Green Fluorescent Protein (GFP) expression in *Escherichia coli* biofilms: effects of surface and nutrient conditions

Ana Azevedo[1], Luciana C. Gomes[1], Rita Teixeira-Santos[1], Gabriel A. Monteiro[2], Filipe J. Mergulhão[1]

1. LEPABE - Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal;
2. iBB - Institute for Bioengineering and Biosciences, Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, 1049-001 Lisboa, Portugal.

Escherichia coli biofilms have been studied as a possible platform for the production of recombinant proteins. Previous studies conducted by our research group have shown that high specific concentrations of enhanced Green Fluorescent Protein (eGFP) could be obtained in *E. coli* biofilms, even before any optimization of the cultivation conditions [1]. This work intends to assess the effect of different surface materials and culture media in both *E. coli* biofilm formation and eGFP production in order to find the optimal combination of these two parameters originating the maximum eGFP yield. *E. coli* JM109(DE3) cells transformed with the pFM23 plasmid carrying the eGFP gene were used and the influence of the surface was assessed. Three surface materials with distinct physicochemical properties - stainless steel (SST), polyvinyl chloride (PVC), and silicone rubber (SIL) – were used combined with three culture media with different sources of carbon and nitrogen - Lysogeny broth (LB), Terrific broth (TB), and M9ZB broth. The physicochemical properties of the surfaces were first assessed by Atomic Force Microscopy (AFM), Scanning Electron Microscopy (SEM), and contact angle determination. Then, the optimization assays were performed using 12-well plates with the different surface materials placed on the bottom of the wells and incubated at 30 ºC under controlled hydrodynamic conditions for 9 days. Biofilm development was evaluated by Colony-Forming Units (CFU) for culturability assessment and Optical Coherence Tomography (OCT) for biofilm thickness measurements, while the eGFP expression was monitored by epifluorescence microscopy [2]. Preliminary results revealed that biofilm formation was higher in PVC surfaces when compared to SST and SIL, regardless of the culture medium used. Additionally, using PVC and TB medium seems to be the most advantageous condition to obtain the highest specific eGFP production in biofilms. These optimization tests on high-throughput platforms as microplates are essential to establish the best operational conditions to be used in further experiments for the production of recombinant proteins and other high-added value compounds on larger-scale biofilm platforms.

Keywords: recombinant protein expression, Green Fluorescent Protein, biofilm, *Escherichia coli*.

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Preventing biofouling formation in RO membrane systems with biocidal macroparticles

Ana C. Barros, Ana Pereira, Luís F. Melo

LEPABE – Laboratory for Process Engineering, Environment, Biotechnology and Energy, Department of Chemical Engineering, Faculty of Engineering of the University of Porto, Rua Dr. Roberto Frias, s/n, 4200-465 Porto, Portugal

High water quality and quantity demand purification strategies to reduce the discharge of biocides in water streams. Reverse osmosis (RO) membrane systems aim to be a biocide-free water treatment technology, but they face operational and maintenance costs associated with microbial accumulation, also known as biofouling.

To overcome such drawbacks, the present work proposes to: (i) functionalize aluminium oxide (Al_2O_3) pellets with an antimicrobial agent (benzalkonium chloride-BAC) after a surface activation process using DA-dopamine; (ii) characterize the particles in terms of thermal stability, textural and structural properties, surface charge, and chemical composition; and (iii) assess their efficacy against *Escherichia coli* planktonic cells through culturability and cell membrane integrity analysis. Previous works focused on biocide immobilization on the surface of nano and micro particles. This work used metal oxide millimetric pellets. This larger dimension is important for the operation of large-scale continuous flow particle bed setups.

The successful immobilization of DA and BAC on the Al_2O_3 particles was demonstrated by thermogravimetric analysis, surface area, X-ray diffraction, surface charge and Fourier Transform Infrared Spectroscopy.

The higher particle concentration tested allowed total inactivation of bacterial cells within 5 min. Also, 100% of cell membrane damage was achieved after 15 min for all concentrations. Furthermore, there was some decrease in particles performance upon reuse, meaning that a higher contact time was needed to reach total inactivation, possibly due to partial blocking of immobilized biocide by bacteria adhering to the particles. The overall results support the feasibility of using Al_2O_3-DA-BAC particles as a sustainable pre-treatment strategy in continuous water treatment systems.

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Characterization of cyanobacterial biofilms formed under different conditions

Maria João Romeu, Filipe Mergulhão

LEPABE - Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

Marine bioufouling is an ongoing concern in aquatic environments, leading to ecological, industrial, economic, and health-related problems.

Cyanobacteria play a pivotal role in marine biofilm development since they are early surface colonizers; they are the major components of these communities, and they promote the attachment of macrofouler organisms such as barnacles, anemones, mussels, or clams. Moreover, over the past years, eutrophication and climate changes have been promoting cyanobacterial blooms in aquatic environments associated with benthic mats. Despite their high adaptability ability, few studies were performed on filamentous cyanobacteria due to technical difficulties in studying these photosynthetic organisms.

Several parameters can affect biofilm behaviour in aquatic ecosystems, but surface properties and shear rate assume a critical impact.

The effective management of cyanobacterial growth is vital to restoring ecosystem function, as well as to minimise biofilms impact. Proteomic studies can provide an essential understanding of cyanobacterial adaptation to diverse situations and can also help to find pathways that affect the adhesion and settlement of biofouling organisms. Moreover, they can contribute to the formulation of new antifouling approaches for marine applications.

Biofilms from different cyanobacterial strains were formed on two different surfaces with relevance for marine environments, glass and perspex. These biofilms were developed under two different shear rates, including hydrodynamic conditions found on real aquatic environments.

This study aims to consolidate cyanobacterial biofilm development analysis from molecular (proteomic approaches) to macroscopic methodologies. Among these regular methods to evaluate of cyanobacterial biofilms, chlorophyll a quantification, wet weigh, and biofilm structure parameters by Optical Coherence Tomography is also used to enable the analysis of the biofilm architecture.

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Optimizing CNT loading in antibiofilm composites for future application in urinary tract devices

Marisa Gomes[1], Luciana C. Gomes[1], Rita Teixeira-Santos[1], Manuel F.R. Pereira[2], Olívia S.G.P. Soares[2],* and Filipe J. Mergulhão[1],*

1. LEPABE – Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal
2. Associate Laboratory LSRE-LCM, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias s/n 4200-465 Porto, Portugal
*Correspondence: salome.soares@fe.up.pt (O.S.G.P.S.); filipem@fe.up.pt (F.J.M.)

The increasing incidence of urinary tract infections has motivated the development of effective strategies to prevent microbial adhesion and biofilm formation on urinary tract devices (UTDs). Carbon nanotubes (CNTs) have been largely used to produce antimicrobial and antifouling coatings with applicability in the biomedical field, particularly in the construction of medical devices and implants [1]. Despite their proven antimicrobial properties, their use as coating materials for UTDs is still poorly documented. In this work, CNT/poly(dimethylsiloxane) (PDMS) composite materials containing different CNT loadings were prepared and further tested against *Escherichia coli*, under conditions prevailing in UTDs. In an attempt to improve the antibiofilm properties of the final composites, textural modifications were also introduced on the surface of CNTs by the ball-milling technique. Material characterization included the textural evaluation of CNTs (through N2 adsorption-desorption isotherms) and the assessment of surface morphology (scanning electron microscopy) and hydrophobicity (contact angle measurements). Biofilm analysis was performed by determining the number of culturable and total cells and by confocal laser scanning microscopy. Results revealed that a total reduction of 39, 28 and 22% were achieved for 3, 4 and 5 wt% CNT-O/PDMS surfaces, respectively. However, only the surface with 3 wt% CNT-O loading presented a statistically significant reduction of cell culturability ($p < 0.001$), being the most promising surface for the inhibition of *E. coli* biofilms. Although only slight differences were observed between pristine and ball-milled CNT-O/PDMS composites (regarding CNT textural properties and surface hydrophobicity), the results showed that a decrease in the number of culturable cells for the ball-milled samples of around 24% when compared to pristine CNT-O. Additionally, the textural modifications induced by ball-milling treatment proved to be effective in inhibiting biofilm formation, reducing the amount of biofilm per surface area, biofilm thickness and surface coverage in 31, 47 and 27%, respectively (compared to not ball-milled surfaces)[2].

Keywords: carbon nanotube composites; poly(dimethylsiloxane); *Escherichia coli*; antibiofilm activity; urinary tract devices.

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Probiotics: a Novel Approach to Fight Biofilms in Urinary Tract Devices

Fábio M. Carvalho, Luciana C. Gomes, Rita Teixeira-Santos and Filipe J. M. Mergulhão

LEPABE - Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

Urinary tract infections (UTIs) are considered the most common bacterial infections worldwide, with a great impact on patients and healthcare systems. About 80% of urinary tract infections (UTIs) are associated with catheter or stent insertion. Urinary tract devices (UTDs) are particularly susceptible to bacterial contamination, which can lead to the development of biofilms on the inner and/or outer surfaces of the device. The presence of biofilms causes numerous problems in the biomedical field, interfering with clinical therapy, as well as persistent infections involving various indwelling medical devices. The primary treatment of biofilm-related infections includes the use of antibiotics, but the growing resistance of pathogens has led to a poor response to antibiotic therapy. Novel technologies to prevent biofilm formation on medical devices are being developed, however, the pursuit for novel and more effective antibiofilm strategies continues.

Inhibition of biofilm formation using probiotics is an attractive approach that has received significant attention in the last years. Probiotics and their metabolites have been described as having the ability to displace adhering uropathogens and inhibit microbial adhesion to UTD materials. This work aimed to evaluate the effect of pre-formed *Lactobacillus plantarum* biofilms on the adhesion of *Escherichia coli*, the most frequent bacterium isolated from biofilms developed in UTDs. Biofilms of *L. plantarum* were formed on silicone coupons in 12-well plates and the *E. coli* suspension was placed in contact with them up to 24 h in artificial urine medium and under quasi-static conditions in order to mimic the biofilm formation on the extraluminal part of UTDs placed inside the bladder, where the shear stress is close to zero. Colony-forming unit (CFU) counts were used to monitor the uropathogen biofilm formation, as well as the culturability of *L. plantarum* biofilms over time. This study showed a reduction of about 1.6 log on *E. coli* culturability after 12 h of contact with the pre-formed probiotic biofilms formed on the silicone surface, suggesting the use of probiotic cells as potential antibiofilm agents for urinary applications.

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Bacterial detection using NAM-FISH and Spectral imaging

Andreia S. Azevedo[1,2], Ricardo Fernandes[1,3], Carina Almeida[1,3,4], Nuno F. Azevedo[1]

1. LEPABE - Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal;
2. i3S, Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal; IPATIMUP - IPATIMUP, Institute of Molecular Pathology and Immunology of the University of Porto, Porto, Portugal;
3. INIAV, IP- National Institute for Agrarian and Veterinary Research, Rua dos Lagidos, Lugar da Madalena, Vairão, Vila do Conde, Portugal;
4. CEB - Centre of Biological Engineering, University of Minho, Braga, Portugal.

One of the most well-established molecular biology techniques for the spatial location of microorganisms is Fluorescent *in situ* hybridization (FISH), that is typically based on the hybridization properties of rRNA with a labelled-probe specifically designed for this purpose. FISH has become increasingly important in clinical diagnosis and in the characterization of complex biofilms communities. However, an important limitation of FISH techniques is the low multiplex capability which is mainly related with 1) the number of distinguishable targets and 2) the difficulty of having probes working perfectly at the same hybridization conditions. Regarding the limited number of targets (1), this is due to the use of filters in fluorescence image acquisition which limits to 2 or 3 color channels the number of fluorophores that can be simultaneously differentiated. To address this issue, new approaches based on spectral imaging, are emerging. In these approaches, instead of looking to the colors being emitted by the sample, the spectral composition (spectrum shape) of each pixel is recorded. That spectrum will then be matched to a reference library to attribute the final composition of the image. For dealing with the probes hybridization conditions (2), our team has been using nucleic acid mimics (NAMs), such as such as, locked nucleic acid (LNA) and 2'-O-methyl-RNA (2'OMe) on FISH. Both LNA and 2'OMe probes, offer higher design flexibility, meaning that with LNA/2'OMe probes fine-tuning is possible by intercalating LNA and 2'OMe monomers. This would allow for a thorough control of the thermodynamic parameters, facilitating multiplex approaches.

Considering the high potential of spectral imaging and the enhanced properties of NAMs, we are combining these strategies to develop a novel color-coded FISH methodology that allows multiplexed and robust detection/location of microorganisms. We intend to implement and validate it as an advanced characterization technique for complex microbial populations. In what concerns to microbial populations, we are already developing multiplex spectral imaging approaches for characterizing the gastric microbiota associated with the presence of *Helicobacter pylori*.

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TOWARDS MORE REPRODUCIBLE BIOFILM EXPERIMENTS

J. Allkja[1,2,3], A.E. Parker[2,3], DM. Goeres[2,3], NF. Azevedo[1,3]

[1]LEPABE, Department of Chemical Engineering, FEUP, Porto, PT, [2] CBE, Montana State University, Bozeman, MT, USA, [3]on behalf of the PRINT-AID ring trial group

Lack of reproducibility among published studies is one of the biggest issues facing science today. Different factors contribute to this e.g., selective reporting, unreliable methods, and lack of data sharing among many others. This project uses different approaches to help us get closer to the goal of reproducible biofilm research.

THE GUIDELINE: Minimum information guidelines instruct authors and reviewers on the necessary information that a manuscript should include for experiments to be clearly interpreted and independently reproduced. An international consortium was consulted to create "Minimum information guideline for spectrophotometric and fluorometric methods to assess biofilm formation in microplates" within the MIABiE framework. (http://miabie.org/introduction.php) The intention of the guideline is to improve reporting for these methods and as a result reproducibility.

EVALUATION OF BIOFILM METHODS: A ring trial was performed in 5 laboratories to evaluate the reproducibility of three microplate-based biofilm quantification methods: plate counts, resazurin, and crystal violet. An interlaboratory (ILP) protocol was developed divided into three steps: biofilm growth protocol, biofilm treatment and biofilm assessment. Experiments were divided into control (steps 1 and 3) and treatment (all three). For treatment experiments, the efficacy of sodium hypochlorite (NaOCl) killing *S. aureus* biofilms was evaluated. Control experiments showed that crystal violet was the most reproducible method with the lowest standard deviation (SD). (Table 1) In the treatment experiments, plate counts had the best reproducibility with respect to responsiveness (SD/slope), making it the more reliable method to use in a disinfectant efficacy test. (Table 2)

Table 1. Summary of results for the STM control data for each method.

Method	Mean Log ± SE	Units	Reproducibility SD
Plate count	7.32 ± 0.40	CFU/well	0.92
Resazurin	0.71 ± 0.22	µg/mL	0.53
Crystal violet	1.13 ± 0.19	µg/mL	0.44

Table 2. Summary of reproducibility with respect to responsiveness for each method.

Method	SD/slope
Plate count	0.98
Resazurin	1.15
Crystal violet	3.22

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DATA SHARING: The data collected from the ring trial is available on the Zenodo repository https://doi.org/10.5281/zenodo.4450073.

CONCLUSIONS: The reproducibility issue is complex and multifaceted. The work presented in this abstract offers possible solutions to tackling this problem in biofilm research. The guideline and ring trial provide a better understanding of microplate methods and an option for data comparability across labs. Furthermore, data sharing can help improve communication and dissemination in the field.

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Drawing Lessons from Nature to Develop Antifouling Surfaces for the Food Industry

Luciana C. Gomes[1*], Kathryn A. Whitehead[2] and Filipe J. Mergulhão[1]
* luciana.gomes@fe.up.pt

1. LEPABE - Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal;
2. Microbiology at Interfaces, Department of Life Sciences, Manchester Metropolitan University, Manchester, UK.

Equipment surfaces in the food industry are usually contaminated by microorganisms, even following cleaning and disinfection. They can grow in the form of biofilms, which are potential contamination sources of finished products, reducing their shelf life and causing foodborne diseases, besides increasing maintenance costs and decreasing operational efficiencies.

The dynamics of microbial adhesion to a food contact surface may be affected by (i) the surface topography, chemistry, and physicochemistry, (ii) environmental conditions such as the presence of other microorganisms, and (iii) processing factors such as fluid velocity and shear force. In previous works, we have shown that the surface material is one of the parameters with the strongest impact on biofilm onset [1, 2]. Therefore, our interest is focused on the development of surfaces that either prevent or reduce bacterial adhesion due to an unfavorable topography or chemistry (antifouling surfaces) or contain compounds that are antibacterial and act against attached cells.

Biomimetic self-cleaning and antifouling surfaces have shown to be an attractive approach for the prevention of bacterial attachment and subsequent biofilm formation since the modification of surface topography may enable the control of the contact area between the surface and the cell. This study developed a biomimetic wax surface using a moulding technique that emulated the topography of the self-cleaning *Gladiolus hybridus* (Gladioli) leaf [3]. A comparison of topographies was performed for unmodified wax surfaces (control), biomimetic wax surfaces, and Gladioli leaves using Optical Profilometry and Scanning Electron Microscopy. The results demonstrated that the biomimetic wax surface and Gladioli leaf had similar surface roughness parameters, but the water contact angle of the Gladioli leaf was significantly higher than the replicated biomimetic surface. The self-cleaning properties of the biomimetic and control surfaces were compared by evaluating their propensity to repel *Escherichia coli* and *Listeria monocytogenes* attachment, adhesion, and retention in mono- and co-culture conditions. When the bacterial assays were carried out in monoculture, the biomimetic surfaces retained fewer bacteria than the control surfaces. However, when using co-cultures, the cell numbers were only reduced on the biomimetic surfaces following retention assays. These preliminary results provide valuable information into the antifouling physical and chemical control mechanisms found in plants, which are particularly appealing for engineering purposes.

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DEVELOPMENT OF MULTIFUNCTIONAL ANTIMICROBIAL SUPRAMOLECULAR BIOMATERIALS

M. Riool[1,2], M.G.J. Schmitz[2,3,4], Leonie de Boer[1], P.Y.W. Dankers[3,4,5], S.A.J. Zaat[1]

[1]Department of Medical Microbiology and Infection Prevention, Amsterdam institute for Infection and Immunity, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands, [2]Dutch Polymer Institute (DPI), Eindhoven, The Netherlands, [3]Institute for Complex Molecular Systems, [4]Laboratory for Chemical Biology and [5]Laboratory for Cell and Tissue Engineering, Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, The Netherlands

INTRODUCTION: The use of biomaterials inside the body always entails the risk of infection. This risk might even be higher in *in situ* tissue engineering applications. Since the porous scaffold materials can form a niche for invading bacteria, the intended *in situ* production of novel tissue may be severely compromised by infection. Therefore, we aim to develop a new polymeric supramolecular scaffold material, exerting two important functions: preventing microbial adhesion and thereby preventing biofilm formation, and inducing endogenous (eukaryotic) cells to regenerate the body.
METHODS: In our research, supramolecular contact-killing materials based on antimicrobial peptides (AMP) are developed. A special class of supramolecular biomaterials are based on fourfold hydrogen bonding 2-ureido-4[1H]-pyrimidinone (UPy) moieties. The supramolecular base material consists of an UPy end-functionalization polycaprolactone (*i.e.* PCLdiUPy). These UPy-materials can be functionalized via a modular approach in which the UPy-base material is mixed with UPy-modified additives[1]. The antimicrobial activity is introduced via UPy-functionalized AMPs, using SAAP-148 or TC84, synthetic derivatives of LL-37[2] and thrombocidin-1[3], respectively. The regenerative activity is introduced via an UPy-functionalized heparin binding peptide (UPy-HBP). The peptides were synthesized by manual Fmoc-based solid phase peptide synthesis. Solid polymer films were prepared by drop-casting PCLdiUPy with UPy-SAAP-148 or UPy-TC84 on glass coverslips. The antimicrobial activity of the UPy-AMPs in solution and when incorporated in the drop-casted samples was evaluated against *Escherichia coli* ESBL and *Staphylococcus aureus* JAR060131 and LUH14616 (MRSA) and *Acinetobacter baumannii* RUH875 using the LC99.9 (*i.e.* the lowest concentration killing at least 99.9% of the inoculum) and the JISZ2801 surface antimicrobial assay, respectively. Moreover, the cytotoxicity of these AMPs was tested against human dermal fibroblasts.
RESULTS: Coupling of the UPy-linker to SAAP-148 did not influence its antimicrobial activity in solution. For the solid drop-casted materials, incorporation of 5 mol% UPy-SAAP-148 is sufficient for killing all 4 bacterial strains tested. This indicates that the peptide remains active after immobilization in the materials. Unfortunately, TC84 loses its antimicrobial activity upon UPy-coupling, both in solution and as a solid. QCM-D adsorption studies revealed that heparin adsorbed to spin coated material films of PCLdiUPy with 5 mol% UPy-HBP mixed via the modular strategy.
Current studies focus on characterization of the UPy-SAAP-148/TC84 and multifunctional biomaterial with XPS, AFM, WCA, zeta potential and leakage experiments to investigate the material properties. Moreover, we assess the *in vivo* efficacy of dip-coated titanium implants with 5% UPy-SAAP-148 in the experimental biomaterial-associated infection mouse model.

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CONCLUSIONS: In conclusion, this modular approach will enable a stable but dynamic incorporation of AMPs, and control of cell adhesion by using cell-adhesive peptides. Ultimately, we aim to use such materials for *in situ* infection-free tissue engineering.

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Development of an early-warning biofouling monitoring system for Legionella prevention in cooling water systems

Tomás Moreira Soares da Costa, Ana Pereira, Luis F. Melo

LEPABE - Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal.

Biofouling, i.e. the undesirable accumulation of a biotic deposit on a surface, is a common problem found in the pipes of cooling systems of many industries causing major economic losses, by damaging expensive equipment, inducing loss of production or increasing maintenance costs. Furthermore, the production of possibly toxic metabolites and the presence of opportunistic pathogens, such as Legionella pneumophila, growing within biofilms raise public health concerns. Therefore, the development of online, real-time monitorization systems to assess the tendency of formation and removal of biofouling in surfaces commonly found in cooling water systems is crucial to prevent biofouling and its consequences.

This project will use the monitoring technique - Mechatronic Surface Sensor (MSS) - that takes advantage of the vibration properties of surfaces, assessing the effect that the biofilm formation/removal has on the vibration propagation. As a first objective, different hydrodynamic conditions (laminar flow vs turbulent flow) will be tested simulating the conditions felt across cooling tower systems. Since, these conditions may be dissimilar in different areas of the pipes, favouring biofilm attachment and growth to different extensions, the “worst-case scenario” conditions (a set of conditions that mostly favour the biofilm formation) will be established. Also, the impact on biofilm formation/removal and on the MSS outputs of different substances (iron, calcium carbonate or aluminosilicate clays), known to be present in cooling systems and relevant components of the biofouling layers, will be evaluated (under the pre-defined worst-case conditions). The ultimate goal is to use a mathematical modelling approach to combine the MSS information with the different biofouling properties on the definition of the biofouling potential indicator (BPI). These BPI will provide an accurate information about the deposits build-up rates as well as on their structural changes.

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How an integrated monitoring model can improve Legionella management in water systems

Ana Pereira, Luis F. Melo

LEPABE - Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal.

Legionellosis (the life-threatening disease caused by Legionella) is known to be preventable if proper measures at engineered water systems are put in practice. Despite the efforts to improve control approaches, Legionella prevention remains one of the most challenging issues in the water treatment industry. Legionellosis incidence is on the rise and expected to keep increasing as global challenges become a reality. This puts great emphasis on prevention, which must be grounded in strengthened Legionella management practices. In fact, one of the main bottlenecks of current Legionella real-field prevention is the overreliance on discrete water sampling results, very often disregarding the role of biofilms as critical spots for Legionella proliferation (in association or not with protozoa). Changing this paradigm requires an integrated monitoring approach which can simultaneously combine discrete and continuous (online, real-time) information about water and biofilm. Under this scenario, online biofilm monitoring tools, implemented under worst case scenario conditions, can provide important early-warning information (e.g. about the deposits build-up kinetics, unexpected sloughing-off events and about the efficacy of countermeasures, like disinfection procedures. This early-warning information is very relevant to trigger specific calls for action.

In this work we aim to bridge three of the pillars previously mentioned to strengthen Legionella control practices at real-field system:

a) address the fundamental relations between Legionella and biofilms to the external system-specific conditions (hydrodynamics, temperature, other water components like iron or inorganic compounds);

b) take advantage of online surface monitoring, exploring the potentiality of the Mechatronic Surface Sensor (MSS) tool, to gain continuous information about the biofilm interaction with the surface – build-up/ removal trends and its structural changes over time;

c) provide a matrix for the definition of worst-case scenario conditions, in which ‘proadhesion’ surfaces can play a decisive role (not only for online measurement, but also for routine sampling analysis).

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NON PRESENTED WORK

Photoactive surfaces of polymer nanocomposites with antimicrobial properties

E. Skoura[1], P. Boháč[1,2], J. Bujdák [1,3]

[1]Slovak Academy of Science, Institute of Inorganic Chemistry, Bratislava, Slovak Republic; [2]Centre of Excellence for Advanced Materials Application (CEMEA), Slovak Academy of Sciences, Bratislava, Slovak Republic; [3] Comenius University in Bratislava, Faculty of Natural Sciences, Department of Physical and Theoretical Chemistry, Bratislava, Slovak Republic

Synthetic saponite (Sap) was modified with hexadecyltrimethylammonium (HDTMA) cations leading to highly hydrophobic material. Subsequently, the organoclay was functionalized with variable amounts of methylene blue (MB). The suspensions of Sap/HDTMA/MB were filtered through Teflon membranes to prepared thin films. The synthesis of nanocomposites with polycaprolactone (PCL) with the functionalized organoclays was performed via melt diffusion at the interface of the film and the polymer. Pristine silicate and modified materials, as well as the final nanocomposites with the modified surface, were characterized by a combination of analytical techniques including UV-Vis absorption and fluorescence spectroscopy.

Some of the prepared specimens exhibited high photoactivity. The optical properties and photoactivity of the dye reflected the concentration of the dye in the materials. The samples with a higher concentration of methylene blue exhibited a higher tendency to form H-aggregates which led to the quenching of luminescence. In the samples of lower concentrations of methylene blue, the formation of monomers is more likely to occur. The absence of any crystalline phase in the XRD diffraction patterns of modified Sap indicates that the Sap layers were exfoliated and dispersed in the surface of the polymer.

The samples with high concentration of MB shows antimicrobial activity against the strain of *S aureus* CCM 3953.

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Functional Hybrid PVD-Coated/Textured Anti-Microbial Surfaces

Niall Hall, Peter Kelly, Kathryn Whitehead and Tomasz Liskiewicz

Manchester Metropolitan University, UK

As has been reported widely throughout the literature, there is an increasing problem in modern medicine, to do with the rise in the number of antibiotic resistant bacterial strains. This is a major problem for medical implants, specifically implants such as the pin-tract implant. These antibiotic resistant bacteria are speculated to cause 2.4 million deaths worldwide by 2050. This problem can be addressed via the implementation of antimicrobial surfaces, or surface textures that reduce the adhesion of the bacteria. This research project aims to combine the two methods and optimise their performance. We have carefully selected specific materials, deposition techniques and textures for these reasons. During this project, we will manufacture the surfaces and characterise them to further understand their properties. Titanium nitride, with silver nanoparticles being co-deposited are the materials that have been chosen for this objective, the materials will be deposited onto 316 medical grade stainless steel as the control, via the physical vapour deposition (PVD) method and the bacteria that will be investigated is *Staphylococcus aureus*. *S. aureus* has been chosen specifically, as it has been found to be one of the most prevalent infection causing bacteria to do with the chosen application. The 316 medical grade stainless steel was also chosen due to it being the most common metal used for the application. The chosen application for this project is the coating of external fixation devices, which are implants that are utilised when a bone is fractured and requires external stabilisation and alignment. Initially the stainless steel will be laser patterned by exposing the surface to femtosecond pulsed laser irradiation, via the GF Machining Solutions Laser Model P400 U, which will produce a precisely controlled surface topography that will aim to exhibit the desire anti-biofouling properties whilst also providing the desired corrosion and wear resistance properties. The patterned surfaces will then be coated via the aforementioned PVD method, Once deposited the surface will undergo a series of analytical techniques including, contact angle measurement, atomic force microscopy (AFM), scanning electron microscopy (SEM), energy dispersive x-ray spectroscopy (EDX), and Fourier transform infrared spectroscopy (FTIR), which will allow us to understand the chemical and physical properties of the surfaces.

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Heterologous protein expression in *Escherichia coli* biofilms

A. Soares*, L.C. Gomes, F.J. Mergulhão

LEPABE – Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias s/n, 4200-465 Porto, Portugal
*up201711436@edu.fe.up.pt

Biofilms are mostly known for their negative effects on human health and industrial productivity. However, they have beneficial use in wastewater treatment and are being tested for the production of valuable compounds such as solvents, organic acids and enzymes. The biological organization of biofilms provides them with many advantages over the suspended cells, including high cell density and protection against hostile conditions. In the last years, the growth of the biotechnology industry led to improvements in the production process of recombinant proteins. Despite the production of a large number of proteins at a commercial scale, the production of recombinant proteins is still a challenge. Thus, a strategy that combines the production of recombinant proteins with bacterial biofilms can be advantageous for different biotechnology industries.

Escherichia coli remains one of the most widely used bacteria for heterologous protein production due to its inexpensive and fast high-density cultivation, well-characterized genetics, and availability of a large number of cloning vectors. Our group has already demonstrated that *E. coli* biofilms grown in a flow cell system were able to produce about 30 times more enhanced green fluorescent protein (eGFP) than their planktonic counterparts. Even without optimization of cultivation conditions, attractive productivity was obtained, indicating that biofilm cultures can be used as an alternative form of high cell density cultivation (HCDC) [1].

The main goal of this study is to optimize the production strategy of eGFP in the flow cell system by increasing both the specific and volumetric production of this model protein in *E. coli* biofilms. For that, the influence of environmental factors (nutrient medium composition, surface properties, and hydrodynamic conditions), as well as the effect of chemical induction (induction time and inducer concentrations) in the eGFP expression are being studied. The first results showed that biofilm growth on polyvinyl chloride (PVC) surfaces was favored in M9ZB medium when compared with Lysogeny broth (LB). However, the number of eGFP-expressing cells was higher in LB for both planktonic and sessile states (2-fold and 7-fold, respectively). Also, the plasmid copy number in biofilm cells was slightly higher in LB medium.

Reference:

1. Gomes LC and Mergulhão FJ. Heterologous protein production in *Escherichia coli* biofilms: A non-conventional form of high cell density cultivation. Process Biochemistry, 2017. 57: p. 1-8.

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The role of mineral-facilitated HGT in the environmental dissemination of antibiotic resistant genes

Kamille Clasen and Karina K. Sand

Section of GeoGenetics, Globe institute, UCPH

Normally extracellular DNA quickly degrades in environments outside the cells. Some minerals have however been shown to bind and stabilise extracellular DNA molecules and such minerals can be important in the environmental spread of genes e.g. ARGs to possible pathogenic bacteria. Biofilms is found various places in nature hereunder on minerals in sediments and one of the emerging properties of biofilm is enhanced HGT. Based on this knowledge we predict that minerals and sediment can be previously unrecognised hotspots for transfer of ARGs to bacteria through mineral-facilitated HGT.

My project seeks to investigate the phenomenon of mineral-facilitated gene transfer and contribute with knowledge that can be used to build a conceptual model for predicting natural sediment deposits and environments were mineral facilitated transfer of genes takes place. I will investigate the factors important for mineral-facilitated transfer hereunder DNAs binding to mineral surfaces and mineral-bacterial affinities. I will be using a gram-positive *B. subtilis* system and a range of minerals such as different clays and iron oxides and I will conduct my experiment in different ionic solution.

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The effect of chitosan-based surfaces to prevent single- and dual-species biofilm formation on implantable medical devices

Rita Teixeira-Santos, Marta Lima, Luciana C. Gomes and Filipe J. Mergulhão

LEPABE - Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

Implantable medical devices (IMDs) are commonly used in clinical practice for both diagnosis and therapeutic purposes. However, their use is associated with several clinical complications, including the occurrence of implant-associated infections (IAIs), which account for 25.6% of all hospital-acquired infections. Despite IAIs being often caused by *Staphylococcus* spp. (67.0%), many other pathogens may be responsible for these infections, including *Enterococcus* spp., *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida* spp. In fact, IMDs are very susceptible to microbial adhesion and, consequently, biofilm formation. The resistance of established biofilms to conventional therapies poses serious clinical challenges and has led to the development of new strategies to prevent IAIs. Chitosan (CS) is a natural polymer that has been widely used in the medical field due to its antimicrobial properties. Hence, the present study aims to evaluate the performance of polylactic acid (PLA) surfaces (a common material used for the construction of IMDs) immobilized with different molecular weight (Mw) chitosan to inhibit single- and dual-species biofilm formation by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. For this purpose, biofilms were developed in 12-well microtiter plates containing PLA (control) and four CS-based surfaces with different Mw (CS1 of 294 kDa, CS2 of 186 kDa, CS3 of 129 kDa and CS4 of 61 kDa). Bacterial suspensions of *S. aureus* and *P. aeruginosa* were used to form single and dual-species biofilms. Results demonstrated that CS-based surfaces were able to inhibit the development of single- and dual-species biofilms by reducing the number of total, viable, culturable, and viable but nonculturable cells up to 95%, being their activity dependent on chitosan Mw. Moreover, the effect of CS-based surfaces on the inhibition of biofilm formation was corroborated by biofilm structure analysis using confocal laser scanning microscopy.

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Eradication of bacterial biofilm using a layered silicate functionalized with phloxine B

Nitin Chandra Teja Dadi[1], Matúš Dohál[1], Veronika Medvecká[2], Juraj Bujdák[3,4], Kamila Koči[1], Anna Zahoranová[2], Helena Bujdáková[1*]

[1]Comenius University in Bratislava, Faculty of Natural Sciences, Department of Microbiology and Virology, Bratislava, Slovak Republic
[2]Comenius University in Bratislava, Faculty of Mathematics, Physics and Informatics, Department of Experimental Physics, Bratislava, Slovak Republic
[3]Comenius University in Bratislava, Faculty of Natural Sciences, Department of Physical and Theoretical Chemistry, Bratislava, Slovak Republic
[4]Department of Inorganic Chemistry of SAS, Bratislava, Slovak Republic

The surface modification of medical devices and photodynamic inactivation (PDI) are highly promising approaches that exhibit the immense potential to eradicate biofilm-related infections. This research aimed to prepare a hybrid film based on clay mineral saponite (Sap) with the immobilized non-toxic photosensitizer phloxine B (PhB). Sap consists of high cation exchange capacity, exfoliating into nanolayers, and modifying different surfaces. A Sap dispersion of 1 g/ L was deposited evenly over a cover glass. The surface charge was modified by adding a cationic surfactant trimethyloctadecylammoniumbromide (ODTMA), and finally, 0.05 mM PhB was entrapped into Sap, creating hybrid films. Physicochemical characterization was performed for these hybrid films revealing the X-ray diffraction of the films confirmed the intercalation of both the surfactant and PhB molecules in the Sap film. The fluorescence spectra measurements demonstrated the retainment of photoactivity by PhB. The water contact angles and measurement of surface free energy demonstrated the hydrophilic nature of the hybrid films. The antimicrobial assays were conducted by PDI on hybrid films against standard strain and clinical isolates methicillin-resistant bacteria of *Staphylococcus aureus* (MRSA). One group of samples was irradiated (green LED light; 2.5 h) compared to non-irradiated ones. *S. aureus* strains show an apparent reduction in growth from 1-$\log_{10}$ to over 3-$\log_{10}$ compared to the control samples with Sap only. Scanning electron microscopy results agree with antimicrobial assays showing low attachment of cells in hybrid films alsong with clear cell disruption observed in microscopic images. These results demonstrate the positive impact of combination techniques which could be a possible alternative for antibiotics and have immense potential in manufacturing medical devices.

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